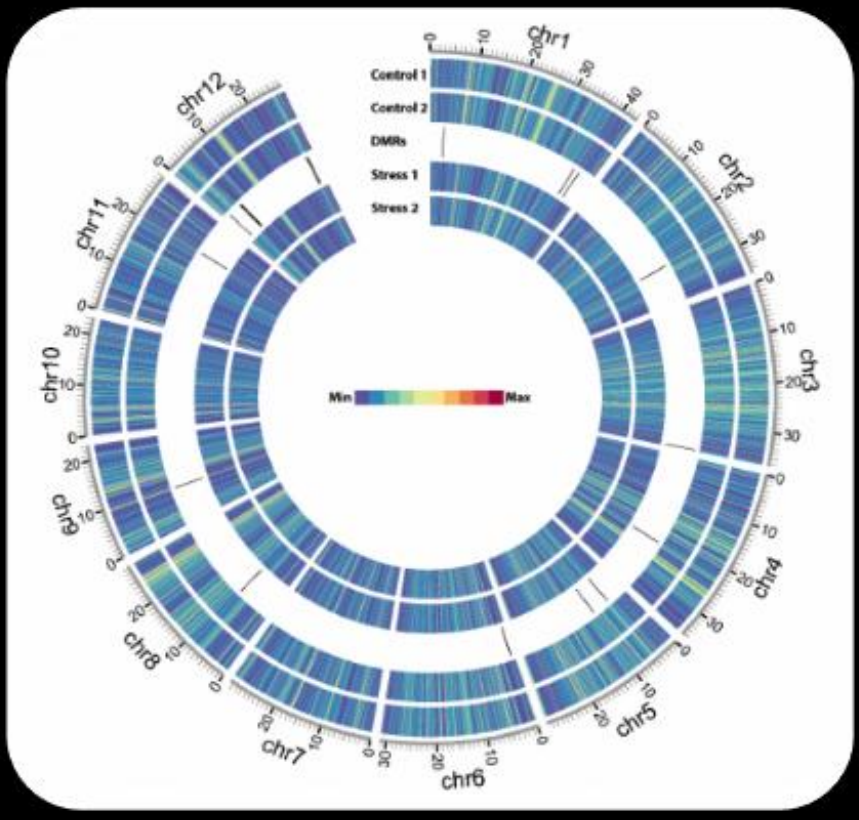
Insights into the epigenetic regulation of the rice genome: the role of DNA methylation and histone modifications in salt stress responses

Liliana de Jesus Duarte Ferreira



Dissertation presented to obtain the Ph.D degree in Biology

Instituto de Tecnologia Química e Biológica António Xavier | Universidade Nova de Lisboa

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Aos meus pais,

pelo amor e apoio incondicionais

If you can't explain it simply,

you don't understand it well enough.

Albert Einstein

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The author of this thesis, Liliana de Jesus Duarte Ferreira, hereby declares to have had active participation in the following research papers:

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This manuscript includes part of the work described in Chapter 1.

Ferreira LJ, Azevedo V, Maroco J, Oliveira MM, Santos AP **(2015)** Salt tolerant and sensitive rice varieties display differential methylome flexibility under salt stress. *PloS ONE* 10(5):e0124060.

This manuscript includes the work described in Chapter 2.

Ferreira LJ, Donoghue MTA, Borges F, Saibo NJ, Martienssen R, Oliveira MM, Santos AP. Identification of Differentially Methylated Regions in a salt tolerant rice variety and their role in gene expression regulation. (in preparation)

This manuscript includes the work described in Chapter 3.

Ferreira LJ, Ravasco S, Figueiredo D, *et al.* Deciphering histone modifications in rice by chromatin immunoprecipitation (ChIP) and *in situ* immunofluorescence. (accepted for publication in the book under the working title *Rice*)

This manuscript includes part of the work described in Chapter 4.

List of abbreviations

3D – Three-dimensional

5-AC – 5-azacytidine

5-mC – 5-methylcytosine

ERF – Ethylene Response Factor

ATP – Adenosine triphosphate

bp – Base pair

BS - Bisulfite

BSA – Bovine serum albumin

cDNA – Complementary DNA

ChIP – Chromatin immunoprecipitation

DNA – Deoxyribonucleic acid

DMR – Differentially methylated region

dS - Decisiemens

DTT – Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

EGTA - Triethylene glycol diamine tetraacetic acid

ELISA – Enzyme-linked immunosorbent assay

FISH – Fluorescence *in situ* hybridization

HAT – Histone acetyltransferase

HDAC – Histone deacetylase

HDM – Histone demethylase

HMT – Histone methyltransferase

M – Molar

Mb – Mega base pair

mM – Millimolar

mRNA – messenger RNA

MSAP – Methylation-sensitive amplified polymorphism

MTase – DNA methyltransferase

PCR – Polymerase chain reaction

PIPES - Potassium piperazine-1,4-bis(2-ethane)sulfonic acid

PTM – Post-translational modification

qPCR – Real time quantitative PCR

rDNA – Ribosomal DNA

RNA – Ribonucleic acid

rRNA – Ribosomal RNA

ROS – Reactive oxygen species

RT-PCR – Reverse transcription PCR

SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis

T-DNA – Transfer DNA

TF – Transcription factor

TSA – Trichostatin A

v/v – Volume / volume

w/v – Weight / volume

WB – Western blot

WT – Wild type

µM – Micromolar

Summary

Plants exhibit complex and integrated strategies to respond to stressful events involving particular signalling pathways, transcription factors and a number of specific stress-responsive genes. In addition, the regulation of genome accessibility is ensured by an efficient network of chromatin organization and epigenetic mechanisms which imply a heritable influence on gene activity that is not associated with changes in the DNA sequence. However, the functional role of these factors in plant response to stressful conditions is still largely unknown and so far direct connections between epigenome regulation and phenotypic plasticity are still poorly explained.

The present thesis addresses the role of the epigenetic regulation of rice responses to salt stress, focusing on DNA methylation and histone modifications. Rice (*Oryza sativa L*.) is the staple food for more than half of the world population and faces specific problems with cultivation under salinity stress, posing a real threat to the sustained production of this cereal.

DNA methylation has been referred as an important player in plant genomic responses to environmental stresses. The analysis of global DNA methylation in different tissues of rice varieties with distinct salt susceptibility showed a global demethylation upon high salt imposition which was particularly evident in leaves. Moreover, the salt tolerant and sensitive rice varieties exhibited differential methylome flexibility, with the salt-tolerant variety Pokkali showing a remarkable ability to quickly relax DNA methylation in salt stress response. The phenotypic evaluation of some parameters related to salinity tolerance, such as root length and biomass, revealed a better performance of the *osdrm2* mutant (defective in a DNA methyltransferase) under stress. All together, these findings emphasize a tissue- and genotype-specificity concerning global DNA methylation levels and suggest that higher methylome flexibility is an important player in salinity tolerance.

A deeper analysis into the methylome profile of leaves of the salt tolerant variety Pokkali, achieved by the MeDIP-seq method, led to the identification of salt stress-specific Differentially Methylated Regions (sDMRs) between control and salt stress samples. The methylation pattern analysis of these sDMRs revealed a loss of methylation in response to salt stress and occasionally the position of the sDMRs, namely their proximity to genes, was correlated with salt stress gene induction, suggesting that sDMRs may have a role in gene expression regulation.

The transcription control of stress responsive genes involves a multilayer regulatory process that includes chromatin structure, histone modifications and transcription factors. Here, we investigated the role and dynamics of specific histone modifications in the regulation of rice *ROOT MEANDER CURLING (OsRMC*, a gene highly induced by salt stress, and very conserved among rice varieties). The analysis of specific histone modification marks by Chromatin Immunoprecipitation (ChIP) in rice leaves revealed a differential enrichment of euchromatic marks depending on the promoter region. Upon salt stress, the chromatin domain where transcription factors bind was particularly enriched in histone modification marks related to euchromatin structure, suggesting a nucleosome repositioning associated with salt stress activation of *OsRMC*.

The knowledge gathered in this work contributes for a better understanding of the mechanisms controlling the plasticity of chromatin and epigenetic networks in plant response/tolerance mechanisms to salinity stress. Research in the field of environmental epigenetics will enhance our knowledge on genome and phenotype regulation and help designing strategies to improve plant adaptation and performance in sub-optimal conditions. This is especially needed, considering the increasing global population and accelerating climate changes, with higher and more variable temperatures, increased soil salinity, severe droughts and flooding.

Sumário

As plantas possuem múltiplas estratégias de grande complexidade para responder a condições de *stress* envolvendo determinadas vias de sinalização, fatores de transcrição e uma panóplia de genes específicos de resposta a stress. A regulação da acessiblidade do genoma depende da organização estrutural da cromatina e de mecanismos epigenéticos que influenciam a atividade de genes sem que haja alterações na sequência do ADN. No entanto, conexões funcionais entre regulação epigenética, plasticidade fenotípica e resposta ao *stress* são ainda pouco compreendidas.

Esta dissertação aborda o papel da regulação epigenética do genoma de arroz em resposta ao *stress* salino, focando-se em particular na análise da metilação do ADN e das modificações das histonas. O arroz (*Oryza sativa L*.) é a base da alimentação de mais de metade da população mundial e apresenta problemas específicos no cultivo sobre *stress* salino, ameaçando a sustentabilidade da produção deste cereal.

A metilação do ADN é extensamente referida como um importante fator nas respostas genómicas das plantas face a *stress*es ambientais. A análise da metilação global do ADN em diferentes tecidos de variedades de arroz com suscetibilidades distintas à salinidade mostrou a ocorrência de uma desmetilação global após imposição de *stress* salino, particularmente evidente nas folhas. Além disso, as variedades tolerantes e sensíveis ao sal evidenciaram diferentes capacidades de flexibilização do metiloma. A variedade tolerante ao sal Pokkali evidenciou uma capacidade notável de rapidamente diminuir a metilação do ADN em resposta ao *stress* salino. A avaliação fenotípica de alguns parâmetros particularmente relacionados com a tolerância à salinidade, nomeadamente o comprimento da raíz e a biomassa, revelaram uma melhor performance do mutante *osdrm2* (knockout numa metiltransferase do ADN) em condições de *stress*. Em

conjunto, estes resultados realçam uma especificidade a nível de tecido e de genótipo no que diz respeito aos níveis de metilação globais, sugerindo que a flexibilidade do metiloma possa ser um factor relevante na tolerância à salinidade.

Uma análise mais aprofundada do metiloma de folhas da variedade de arroz tolerante ao sal por MeDIP-seq, permitiu a identificação de Regiões Diferencialmente Metiladas específicas do *stress* salino (sDMRs) entre amostras de controlo e *stress* salino. A análise do padrão de metilação destas sDMRs revelou uma perda de metilação em condições de *stress* salino e, em alguns casos, a posição das sDMRs, nomeadamente a sua proximidade de genes, foi correlacionada com a indução dos genes pela salinidade, sugerindo um papel regulatório das sDMRs ao nível da expressão génica.

O controlo da transcrição de genes de resposta ao *stress* envolve múltiplos processos reguladores que incluem a estrutura da cromatina, fatores de transcrição e modificações histónicas. Neste contexto, investigou-se o papel de modificações de histonas específicas na regulação transcricional do gene de arroz *ROOT MEANDER CURLING* (*OsRMC*, altamente induzido pelo sal e bastante conservado entre diferentes variedades de arroz). A análise de marcas de modificações de histonas específicas, por imunoprecipitação da cromatina (ChIP) em folhas de arroz revelou um enriquecimento diferencial em marcas eucromáticas dependendo da região do promotor. A região da cromatina onde se liga o fator de transcrição mostrou um enriquecimento em marcas associadas a eucromatina após o *stress* salino, sugerindo que possa ocorrer um reposicionamento dos nucleossomas aquando da ativação do gene *OsRMC* pelo *stress* salino.

Este trabalho contribui para uma melhor compreensão da plasticidade da organização estrutural da cromatina e marcas epigenéticas na resposta/tolerância das plantas ao *stress* salino. Por ajudar a compreender

melhor a regulação genómica e fenotípica, a investigação na área da epigenética ambiental pode ajudar a delinear estratégias para o melhor desempenho das plantas em condições desfavoráveis. Esta investigação é especialmente necessária tendo em conta o aumento da população global e a rápida evolução das alterações climáticas, nomeadamente temperaturas mais altas e variáveis, aumento da salinidade do solo, secas severas e inundações.

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Chapter 1

General Introduction and Research Objectives

Liliana J. Ferreira performed the bibliographic search and wrote this chapter, part of which was submitted for publication as:

Ferreira LJ, Oliveira MM, Santos AP. Chromatin and epigenetics flexibility in plant responses to environmental stresses.

Chapter 1:
General Introduction and Research Objectives

1.1. Chromatin and nuclear architecture have a role in genome regulation

In eukaryotes, the genetic information is encoded by DNA which can have several meters and thus needs to be packaged in order to fit into a physically restricted space that is the nucleus. A high level of compaction and organization is achieved through association of DNA with proteins named histones, forming chromatin (Figure 1). The nucleosome is the fundamental unit of chromatin and is composed by approximately 146 bp of DNA wrapped around an octamer of histones, containing two copies of each of the four core histones (H2A, H2B, H3 and H4) (Kornberg, 1974). Each nucleosome is linked to the next nucleosome through a portion of linker DNA, creating the 10 nm fiber known as the "beads on a string" model (Luger *et al.,* 1997). The length of linker DNA ranges between approx. 20–90 bp and varies among different species, tissues, and even fluctuates within a single cellular genome (van Holde, 1988). The linker histone H1 drives the package of the 10 nm fibers into a solenoid of 30 nm diameter, forming a super helix with 6 nucleosomes per turn (Finch and Klug, 1976; Robinson *et al.,* 2006). In an alternative model the 30 nm fiber is composed by a "zigzag" nucleosome array and the spacer DNA frequently passes through the central axes of the fibre (Schalch *et al.,* 2005; Woodcock and Ghosh, 2010). Higher levels of DNA compaction acting on the 30 nm fibers are normally referred as the large-scale level of chromatin organization which is still a matter of discussion (Woodcock and Ghosh, 2010). The knowledge of the spatial arrangement of chromatin in the 3D interphase nucleus greatly benefited from the emergence of the *in situ* hybridization technique with specific DNA

probes and from the improvement of microscope resolution. Fluorescence *in situ* hybridization (FISH) with chromosome-specific probes started to be successfully applied in animal cells showing that interphase chromosomes occupy distinct territories in the interphase nucleus (Manuelidis, 1984; Lamond and Earnshaw, 1998). More recently, specific regions of chromatin as the lamina-associated domains (LADs), and the topological association chromatin domains (TADs), which are bound together by particular protein complexes, were found in specific nuclear territories (Nicodemi and Pombo, 2014).

The chromatin structure is affected by the incorporation of histone variants such as H2A.Z and CENH3 that greatly influence gene activity and genome structure (Zhang *et al.,* 2005; Deal and Henikoff, 2011; Coleman-Derr and Zilberman, 2012). For example, the histone H3, predominantly present during replication, can be replaced by the histone variant H3.3 in a replication-independent manner in active chromatin regions (Chen *et al.,* 2014). Another divergent form of histone H3, the CENP-A, is exclusively found in centromeric regions, having important functions in regulating the proper segregation of chromosomes (Howman *et al.* 2000; Régnier *et al.* 2005). Similarly, the histone H2A.Z, variant of H2A, is found around gene promoters (Redon *et al.* 2002; Zhang *et al.* 2005).

Chromatin has not only a role in the structural organization of DNA but is also involved in gene expression regulation and in preventing and repairing genomic lesions (Lukas *et al.,* 2011). At cytological level, chromatin can be defined as euchromatin or heterochromatin, which differs mainly on their compaction state and transcriptional potential. Chromatin compaction has been negatively correlated with transcriptional competence since the ability of a gene to be transcribed depends on its accessibility to the transcription machinery. The heterochromatin is highly condensed, transcriptionally inactive and rich in repetitive sequences while euchromatin presents less compaction with irregularly spaced nucleosome arrays, being gene rich and

transcriptionally competent (Heitz, 1928; Fransz *et al.* 2002; Berger, 2007). The chromatin accessibility is mainly determined by the recruitment of non-histone chromatin binding proteins, which recognizes modified histone motifs and exerts its regulatory functions. These functions involve post-translational modifications of histone tails, incorporation of histone variants, nucleosome sliding and remodelling by ATP-dependent remodelling complexes (Rosa and Shaw, 2013).

1.2. Epigenetics: mechanisms and functions

All cells of an organism carry the same DNA sequence but a chromatin-based selective read out of the genome is capable to originate distinct cell types. The study of the transmission of chromatin states without changes of the underlying DNA sequence is the target issue of epigenetic research. The word “epigenetics” was used for the first time by Conrad Waddington (1905–1975) to describe “the branch of biology which studies the causal interactions between genes and their products, which bring the phenotype into being” (Waddington, 1942). In a broad sense, epigenetics can be understood as a bridge between the genotype and phenotype, a group of molecular mechanisms that modulates the final outcome of a *locus* or chromosome without any change on the underlying DNA sequence. Some of the core molecular actors playing epigenetic roles are described below and are represented in Figure 1.

1.2.1. DNA methylation

DNA methylation is the best characterized chemical modification of chromatin. Conventionally, it consists on the binding of a methyl group (CH_3), provided by S-adenosylmethionine (SAM), to the 5- carbon of a cytosine ring (5-mC), in a reaction catalysed by DNA methyltransferases

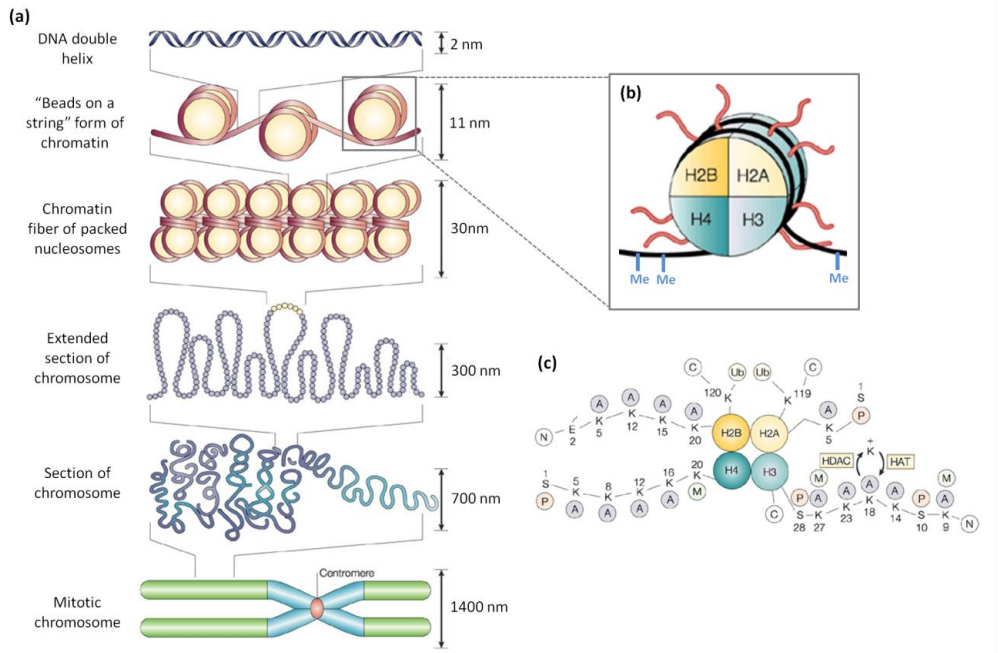

Figure 1: Chromatin structure and epigenetic modifications. Schematic representation of different levels of chromatin organization, from the basic unit of chromatin, the nucleosome, to the highly condensed mitotic chromosome (a). The nucleosome, the fundamental repeating units of chromatin, is schematically shown in (b). The core proteins of nucleosomes are designated H2A, H2B, H3 and H4. Each histone is present in two copies, so the DNA (black) wraps around an octamer of histones. DNA can be epigenetically modified by the addition of a methyl group (Me) to cytosine residues (b). The amino-terminal tails of core histones can be subjected to several post-translational modifications (c). Adapted from Marks *et al.,* 2001; Felsenfeld and Groudine, 2003.

(MTases). The presence of 5-mC in DNA was first detected in the tubercle bacillus (Johnson and Coghill, 1925) and has been considered as the "fifth base" because of the additional variation it can generate (Doerfler, 2006). A more recent discovery is the 5-hydroxymethylcytosine (5-hmC), currently accepted as the "sixth base" (Munzel *et al.,* 2011). Other DNA bases can also be methylated, namely the adenines. In bacteria, the N6-methyladenine (m-6A) has an important role as a defence mechanism against bacteriophage infections. The presence of m-6A is not limited to eubacterial

DNA but also occurs in some archaebacteria and eukaryotic cells, although its role remains largely unknown (Ratel *et al.,* 2006). The methylation does not interfere with the Watson/Crick pairing properties of cytosine but the methyl group, positioned in the major groove of the DNA, can be detected by proteins interacting with DNA.

DNA methylation is present in almost all eukaryotes, but their distribution pattern is quite different among different species. In mammals, genomic DNA methylation is found throughout the genome with the exception of short unmethylated regions called CpG islands (Bird, 2002). Other well-studied model systems are devoid of DNA methylation, as for example, the yeast *Saccharomyces cerevisiae* (Capuano *et al.,* 2014) and the nematode worm *Caenorhabditis elegans* (though the presence of adenine methylation was recently confirmed) (Greer *et al.,* 2015). In fungi, DNA methylation is restricted to repetitive DNA sequences (Selker *et al.,* 2003) and invertebrates have the so-called “mosaic methylation”, comprising domains of heavily methylated DNA interspersed with methylation-free domains (Tweedie *et al.,* 1997). Plants have the highest levels of DNA methylation among all eukaryotes, with up to 50% of methylated cytosines in some species (Montero *et al.,* 1992). One reason for these high amounts of DNA methylation in plants is that it may occur in any sequence context (CG, CHG, CHH, where H = A, C, or T), while in mammals DNA methylation is confined to CG dinucleotides.

There are three distinct DNA methylation pathways with overlapping functions in plants, maintenance methylation, *de novo* methylation and demethylation. The maintenance of CG methylation is under the responsibility of the METHYLTRANSFERASE 1 (MET1) and the chromatin remodelling factor (DDM1) (Finnegan *et al.,* 1996; Jeddeloh *et al.,* 1999), among others. The CHG methylation is controlled by the plant-specific CHROMOMETHYLASE 3 (CMT3), involving also the histone methyltransferase responsible for the H3K9 dimethylation (Bartee *et al.,*

2001; Lindroth *et al.,* 2001; Jackson *et al.,* 2002; Johnson *et al.,* 2007). The DOMAINS REARRANGED METHYLASE 1 and 2 (DRM1/2) maintain DNA methylation at CHH sites (Cao and Jacobsen, 2002; Chan *et al.,* 2004). The DNA methylation pattern can be edited, either by *de novo* methylation or by demethylation, being a unique way to encode information in a stable but reversible manner. *De novo* DNA methylation is established through the RNA-directed DNA methylation pathway (RdDM). The biogenesis of the 24-nt small interfering RNAs (siRNAs) required to target DNA methylation is achieved through the action of the multisubunit plant specific RNA polymerase IV, of the RNA-DEPENDENT RNA POLYMERASE 2 (RDR2) and of the DICER-LIKE 3 (DCL3). Other components of the RdDM pathway are the DNA methyltransferase DRM2, ARGONAUTE 4 (AGO4) and RNA polymerase V, which are needed for the siRNA accumulation (Law and Jacobsen, 2011). DNA demethylation can occur passively when DNA methylation is diluted after DNA replication. In other cases, DNA methylation can be removed through active processes involving the action of 5-methylcytosines glycosylases, namely Repressor of silencing 1 (ROS1), Demeter (DME), DME-like 2 (DML2) and DML3 (in Arabidopsis), normally associated with DNA repair mechanisms (Base Excision Repair-BER), which remove methylated bases and cleave the DNA backbone. The gap is then filled by a DNA polymerase and a DNA ligase (Gehring *et al.,* 2009; He *et al.,* 2011). Although several advances have been achieved on the comprehension of active DNA demethylation, persistent questions remain to be elucidated, namely how DNA glycosylases recognize their targets, since 5-methylcytosine is not a damaged base, pairing perfectly with guanine. In animals, it was recently discovered the Ten Eleven Translocation (TET) family of 5-mC hydroxylases (TET1, TET2 and TET3) that can oxidize and convert 5-mC to three different oxidation products, namely 5-hydroxymethylcytosine (5-hmC), 5-formylcytosine (5-fC) and 5-carboxylcytosine (5-caC) (Tahiliani *et al.,* 2009; Ito *et al.,* 2011). These

findings bring new insights into the process of active DNA demethylation and on their role in gene transcription regulation (Kohli and Zhang, 2013; Scourzic *et al.,* 2015).

DNA methylation is involved in many cellular processes, including silencing of transposable elements, X chromosome inactivation in female mammals, gene imprinting, transgene silencing, and paramutation (He *et al.,* 2011). The methylation of DNA is also a key mechanism to control gene expression. The physical consequences of the methylation of DNA are the obstruction to the binding of transcription factors to the gene and also to the transcription machinery (Tate and Bird, 1993; Choy *et al.,* 2010). Additionally, methylated DNA attracts methyl-cytosine binding proteins, which in turn recruit other chromatin remodelling proteins, including histone deacetylases, leading to chromatin compaction and gene silencing (Nan *et al.,* 1998).

DNA methylation is generally associated with the repression of transposable elements and other repetitive sequences, including the centromeric and pericentromeric regions (Zhang *et al.,* 2006). It is also a powerful mechanism controlling the transcriptional activity of single genes. Similarly to repetitive sequences, DNA methylation in the promoter regions of single genes has been widely correlated with gene repression (Zhang *et al.,* 2006; Li *et al.,* 2007; He *et al.,* 2011). However, the idea that DNA methylation is a hallmark of silenced genes has been consecutively challenged by new discoveries about methylated active genes. For example, 33% of the Arabidopsis genes have CpG methylation in their transcribed regions. Moreover, this gene-body methylation does not shut off gene expression; instead these genes were characterized by moderate level of expression in many tissue types, and many were even classified as ‘housekeeping genes’ (Zhang *et al.,* 2006; Zilberman *et al.,* 2007).

1.2.2. Histone modifications

Histones are highly basic proteins folded into a C terminal globular domain and a flexible relatively unstructured N- tail that protrudes from DNA surface of the nucleosome core particle. Both histone tails and globular domains can be subject to a diverse set of post-translational modifications (PTMs) that modulate their interaction with other chromatin components and hence change the structural and functional properties of chromatin. There are over 60 different residues on histones where modifications have been detected, including methylation of arginine (R); methylation, acetylation, ubiquitination, ADP-ribosylation, and sumoylation of lysines (K); and phosphorylation of serines (S) and threonines (T) (Figure 1). Thus, the PTMs are the basis of a “histone code”, which considerably extends the information of the genetic (DNA) code (Jenuwein and Allis, 2001). However, although the expression “histone code” may help to clarify the need for a specific set of modifications leading to a specific outcome, it hardly reflects a predictable ‘‘code’’ in the strictest sense of the word (Liu *et al.,* 2005). Some authors even claim that post-translational modifications of histones are no different than the post-translational modifications associated with any other proteins in the cell (Schreiber and Bernstein, 2002; Sims and Reinberg, 2008; Lee *et al.,* 2010).

In the last few years, several histone-modifying enzymes have been characterized (Kouzarides, 2007). The histone acetyltransferases have a relatively low specificity, since each enzyme can modify many different lysine residues, although some are specifically limited to certain residues (Kouzarides, 2007). In contrast, histone methyltransferases are highly specific and may be restricted to modifying a single lysine in a single histone (Bannister and Kouzarides, 2005).

The effect of the histone modification is achieved through two ways. One is by affecting higher-order chromatin structure, namely through the disruption of contacts between different histones in adjacent nucleosomes or

between histones and DNA. The other is the recruitment of non-histone proteins carrying specific enzymatic activities, such as remodelling ATPases, which further modify chromatin. Specific histone modifications are associated with various chromatin dependent processes such as the regulation of gene expression and heterochromatin formation. Most studies in plants have focused on the methylation and acetylation of lysine residues on histone H3, namely H3K4me2, H3K4me3, H3K9me2, H3K9me3, H3K9ac, H3K27me, H3K27ac, H3K27me2, H3K27me3, H3K36me3 and H3K56ac (Zhang *et al.,* 2007b; Bernatavichute *et al.,* 2008; Charron *et al.,* 2009; Zhang *et al.,* 2009; Zhou *et al.,* 2010; Roudier *et al.,* 2011). In general, histone acetylation has a quite constant correlation with gene activity, the hypoacetylation being associated with silent genes and the hyperacetylation with active genes. This is explained by the fact that acetylation has a great potential to unfold chromatin, since it neutralizes the basic charge of lysine (Kouzarides, 2007). On the other hand, the methylation of lysine residues can be associated with either gene activation or repression, depending on the residue context. For example, the methylation at H3K9 and H3K36 has a positive effect when it is found on the coding region but can have a negative effect when occurs at gene promoter (Tariq and Paszkowski, 2004; Viejo *et al.,* 2012). Additionally, the H3K9me3 tends to be a mark of active genes in Arabidopsis but in *Drosophila* and mammals it is mainly associated with heterochromatin (Berger, 2007; Kouzarides 2007; Li *et al.,* 2007). The effect of lysine methylation on gene expression also depends on whether the lysine residue is mono-, di- or trimethylated. For example, in Arabidopsis, the H3K4me1 and H3K4me2 are not directly involved in transcriptional activation, but the H3K4me3-containing genes are highly expressed (Zhang *et al.,* 2009).

An important but largely unanswered question is how histone-modifying complexes are recruited to their targets. One mechanism involves the recruitment by transcriptional activators through the action of their protein

domains. For example, bromodomains preferentially bind peptides with acetylated lysines while chromodomains, MBT repeats and PHD fingers can discriminate among lysines that are mono-, di-, or trimethylated (Smith and Shilatifard, 2010). Coactivators and corepressors, as well as the RNA polymerase II and noncoding RNA, are other pathways allowing the recruitment of histone modifying complexes (Smith and Shilatifard, 2010). Other poorly known question is how histone modifications are maintained during mitotic cell division. It is known that during DNA replication there is the recycling of old histones and incorporation of new unmodified histones (Annunziato, 2005; Probst *et al.,* 2009; Margueron and Reinberg, 2010; Alabert and Groth, 2012). This recycling model not only guarantees the correct location of PTMs on newly replicated DNA, but also ensures that the parental histones serve as a blueprint to modify neighbouring new histones, since modifications like H3K9me3 and H3K27me3 can recruit their cognate enzyme and potentially self-propagate (Aagaard *et al.,* 1999; Hansen *et al.,* 2008; Margueron *et al.,* 2009). However, for several other modifications, mass spectrometry analysis showed that new histones had not acquired modifications in order to become identical to the old parental histones (Scharf *et al.,* 2009; Sweet *et al.,* 2010; Xu *et al.,* 2012). Recently, the importance of histone chaperones as coordinators of the recycling of parental and new histones has been described (Gurard-Levin *et al.,* 2014), but still several questions remain to be elucidated.

1.2.3. Interplay between histone modifications and DNA methylation

There is a growing number of evidences for the crosstalk between histone modifications and DNA methylation. For example, H3K9me2 is catalyzed by the histone methyltransferase SUVH4/KYP, which is also required for maintenance of non-CG methylation (Jackson *et al.,* 2002). Simultaneously, the SRA domain of SUVH4 can directly bind to methylated DNA, meaning that DNA methylation is required for recruitment of SUVH4.

Other example of interaction between histone modifications and DNA methylation come from the inability of the DNA methyltransferase CMT3 to bind to the N-terminal tail of histone H3 if the histone tail is not methylated at both H3K9 and H3K27 positions (Lindroth *et al.,* 2004). Therefore, the histone methylation at H3K9 and H3K27 provide a histone code for the recruitment of CMT3 to methylated DNA *loci* (Lindroth *et al.,* 2004; Johnson *et al.,* 2007). Less is known regarding how the DNA demethylation machinery interacts with histone modifications. There are only a few reports in animals showing that elevated histone acetylation triggers DNA demethylation (Cervoni and Szyf, 2001; D'Alessio *et al.,* 2007). Additionally, methyl-binding proteins are a strong link between DNA methylation and histone modifications. In Arabidopsis, the SRA domain of the histone methyltransferase SUVH4/KYP binds to DNA with methylated cytosines in all contexts, but with a preference for CNG and CNN sequences (Johnson *et al.,* 2007). More recently, it was shown that active DNA demethylation is regulated by a methyl-CpG-Binding domain protein (Li *et al.,* 2015). In summary, all these findings point to the existence of a very complex network of chemical modifications acting on DNA and histones to orchestrate several cellular functions.

1.2.4. Methods to decipher epigenetic marks

In the last decade, epigenetics has become one of the most exciting and rapidly expanding fields in biology along with novel methodologies to elucidate epigenetic signatures. Concerning DNA methylation analysis, most techniques relies on a methylation-dependent pre-treatment of genomic DNA in order to reveal the presence or absence of the methyl group at cytosine residues. Currently, there are three main approaches to study DNA methylation namely, the endonuclease digestion, the affinity enrichment of methylated regions and the bisulfite (BS) DNA treatment. The endonuclease digestion is based on the DNA treatment with methylation-sensitive

restriction enzymes. The digested DNA can then be used to access the methylation status of specific sequences by Southern-blot, PCR amplification and hybridization to high-density oligonucleotide arrays. The main drawbacks of this approach are the incomplete enzyme cleavage and the impossibility to determine the context of the methylation (Laird, 2003). The affinity enrichment of methylated regions approach relies on the use of 5-mC specific antibodies or methyl-binding proteins followed by capture of methylated regions by immunoprecipitation. The captured DNA can then be hybridized to a tiling array (MeDIP-chip) or be sequenced (MeDIP-seq). This approach allows a rapid and efficient genome-wide assessment of DNA methylation but it is biased for CG-rich sequences and does not allow a precise determination of the cytosine context (Lister and Ecker, 2009). The bisulfite conversion approach is based on the differential rate at which cytosine and 5-methylcytosine are deaminated by sodium bisulfite since while cytosine is rapidly converted to uracil, 5-methylcytosine remains unaltered (Frommer *et al.,* 1992). Subsequent PCR amplification and sequencing provides high-resolution detection of DNA methylation content and pattern. The BS approach is currently considered the gold-standard method to precisely identify cytosine methylation. The massive advances made in high-throughput DNA sequencing enabled the mapping of DNA methylated sites at single-base resolution throughout the entire genome. Importantly, the criteria for choosing a specific approach must consider the target biological question. The detection of methylation at maximum resolution may not always be necessary, particularly since some studies have revealed a significant correlation between methylation states of cytosines within 1000 bases-long regions (Cokus *et al.,* 2008). Additionally, deciphering DNA methylation at a specific region may be more relevant than uncovering the methylation status of each individual cytosine (Jones and Liang, 2009).

Histone post-translational modifications (PTMs) are generally detected through the use of specific antibodies in a straightforward approach that involves either western blotting or immunoblotting. The quality and specificity of antibodies should be carefully evaluated with respect to cross-reactivity with alternative histone PTMs before experimental application. Currently, there are over 200 commercially available antibodies against different histone PTMs (Egelhofer *et al.,* 2011). Generally, it is important to associate a specific histone PTM with a protein or genomic region. The co-immunoprecipitation/pulldown experiments can be used to study the interaction of a protein or peptide with a PTM, while peptide microarrays allow screening the interaction of probe proteins with multiple peptides simultaneously. If the goal is to determine the genomic *loci* of a PTM, the chromatin immunoprecipitation (ChIP) technique is the best option. Further quantification of the relative proportion of different *loci* with which PTM is associated can be achieved through PCR (ChIP-PCR) or microarray-based techniques (ChIP-chip), depending on the amount of *loci* under analysis. Large-scale enrichment analysis can also be performed using DNA sequencing (ChIP-seq), generating highly comprehensive data with high resolution. However, the ChIP process generates a limited amount of DNA due to the low yield of antibody pull-down, DNA damage during fragmentation and cleavage of DNA-protein complex, forcing to use a considerable amount of samples. Moreover, the ChIP process is not a functional assay and cannot by itself demonstrate the functional significance of a protein or modified histone located at a genomic region of interest (Carey *et al.,* 2009). Recently, the use of microfluidic devices enables a rapid, semi-automated and highly sensitive ChIP assay from a small amount of tissue such as 1000 mammalian cells (Shen *et al.,* 2015).

Epigenetic marks act in a complex combined mode to affect gene expression and this knowledge has triggered a growing interest in studying multiple epigenetic marks simultaneously. ChIP and bisulfite-sequencing

have been combined to ChIP-BS-seq/BisChIP-seq (Brinkman *et al.,* 2012; Statham *et al.,* 2012), NoME-seq (Nucleosome Occupancy and Methylome sequencing) uses *de novo* methylation to simultaneously examine nucleosome occupancy and CpG methylation (Kelly *et al.,* 2012), and CATCH-IT (Covalent attachment of tags to capture histones and identify turnover) estimates nucleosome dynamics, namely the rates of assembly, disassembly and turnover of native nucleosomes (Deal *et al.,* 2010).

The vast diversity of methodologies currently available to analyse epigenetic processes enabled the gathering of valuable information about several epigenetic marks. Nevertheless, the majority of those methodologies are applicable on heterogeneous cell populations and on measuring steady-state levels of the epigenetic marks. Since it is well established that epigenetic processes are highly dynamic and cell- and tissue-specific, techniques allowing the integration of single-cell resolution with *in vivo* analysis should generate more knowledge on the epigenetic regulation of gene expression. In this regard, the immunofluorescence approach presents the advantage of permitting a single-cell resolution analysis of distinct epigenetic marks, e.g. DNA methylation and specific histone modifications. Concerning the *in vivo* analysis, Fluorescence Recovery After Photobleaching (FRAP) is a very accessible technique for analysing the kinetics of molecules in living cells, including histones and other chromatin associated proteins. Another approach to track histone modifications *in vivo* are the genetically encoded, generating fluorescent *m*odification-specific *int*ra-cellular anti*bodies* (mintbodies), which were used to determine the kinetics of specific histone modification (H3K9ac) changes upon treatment with a histone deacetylase inhibitor (TSA) (Sato *et al.,* 2013).

1.3. Epigenetic regulation of stress responses in plants

Plants are constantly exposed to potentially stressful conditions and therefore have developed numerous survival strategies, including developmental, morphological and physiological adaptations. Sensing dangerous situations and rapidly initiating effective responses are essential for successful survival. These responses range from signalling cascades to synthesis of defence compounds, including an intricate and highly regulated gene expression network (Figure 2).

While considerable knowledge has been achieved on physiological stress responses involving individual proteins, genes and transcription factors, much less is known about the effect of stress at whole genome level. The concept of large-scale genomic restructuring events in response to unfavourable conditions was introduced by McClintock, more than thirty years ago (McClintock, 1984). Among these events, changes in heterochromatin have the potential to cause large effects on genome function (Madlung and Comai, 2004).

Epigenetic mechanisms are clearly involved in chromatin modifications induced by stress but the precise mechanisms and molecular interactions are still not fully understood. Distinct types of stress namely tissue culture, pathogen attack, interspecific crosses and abiotic stress have been shown to involve epigenetic regulation (Madlung and Comai, 2004). Concerning abiotic stress, several conditions have been analysed, such as suboptimal temperature, water and nutrient availability, and light conditions. For example, the temperature dependent transposon activation in *Antirrhinum majus* (Coen *et al.,* 1986) was correlated with DNA demethylation under low temperatures (Hashida *et al.,* 2003). Likewise, in *Medicago sativa,* a cold-induced transcriptional activation of multiple copies of a retrotransposon was observed, although in this case not associated with DNA demethylation (Ivashuta *et al.,* 2002). Therefore, transposon activation and transposition is a widespread phenomenon in plants in response to abiotic stress.

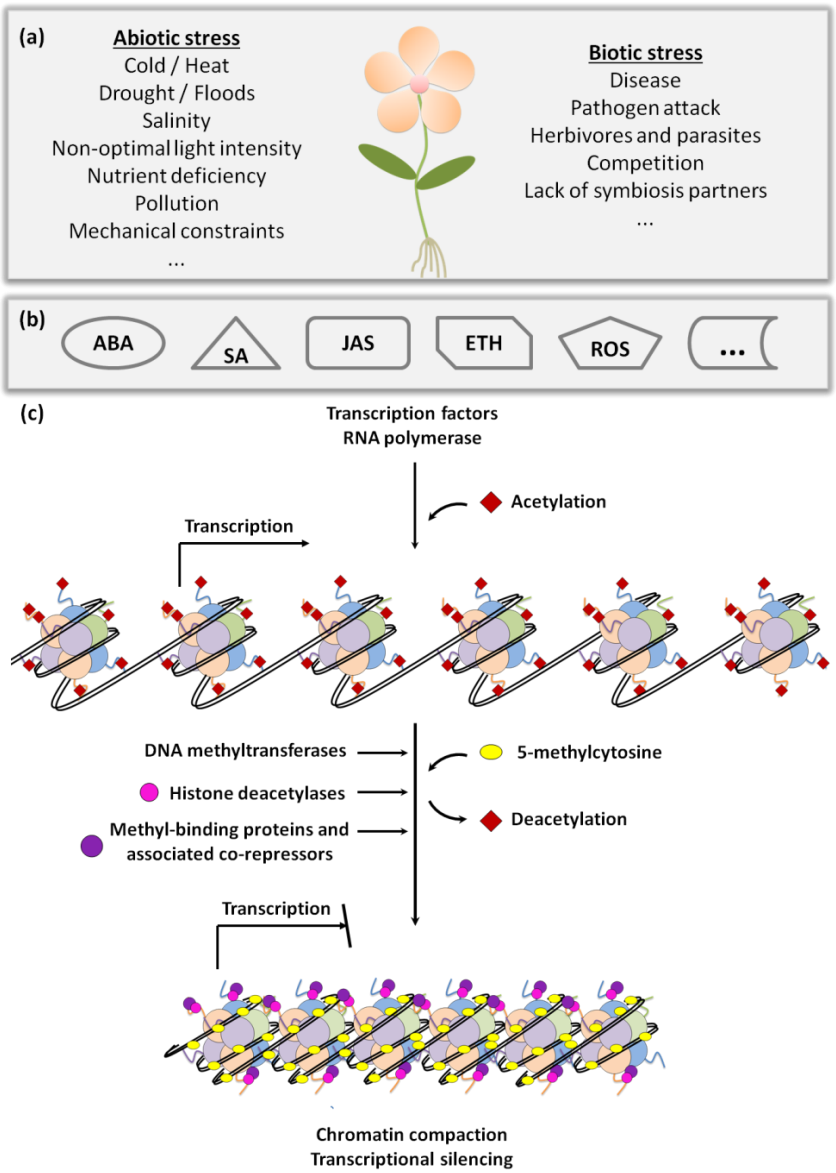

Figure 2: Regulatory network of plant stress responses. Plants can be affected by several abiotic and biotic stress conditions **(a)** and to survive they must respond rapidly and effectively. These responses may include signalling cascades, where several hormones can be involved **(b)**. This leads to transcriptional changes either by involvement of transcription factors or through affecting chromatin conformation via DNA methylation, histone tail modifications, histone variant replacements, or nucleosome rearrangements **(c)** (adapted from Gutzat and Scheid, 2012).

The phenomenon of priming, in which a previous exposure to stress makes a plant more resistant to future stress exposure, is also a general adaptation to stress (Bruce *et al.,* 2007) and recent evidences point to an epigenetic regulation of the priming effect (Luna *et al.,* 2012; Luna and Ton, 2012). Control of gene expression under stress is probably the most studied plant's response to adverse conditions. Since epigenetic mechanisms, namely chromatin modifications, can be mitotically and meiotically inherited, the epigenetic regulation of gene transcription has the potential to cause more permanent changes of gene expression patterns, being a more efficient strategy to cope with stress (Gutzat and Scheid, 2012). Just to provide some illustrative examples, it was shown in Arabidopsis that the histone deacetylases AtHD2C modulates ABA responsive genes, playing an important role in enhancing plant tolerance to salt and drought (Sridha and Wu, 2006). An increase of H3 acetylation was associated with an increased expression of two stress-responsive genes ADH1 and PDC1 after submergence of rice plants (Tsuji *et al.,* 2006). Also in rice, DNA demethylation occurring in response to osmotic stress was found to facilitate proline accumulation by the up-regulation of P5CS and δ-OAT genes (Zhang *et al*., 2013).

1.4. Salinity effects on plants

Soil salinity is a major environmental constraint to crop production worldwide and is expected to rise due to global climate changes and also as a consequence of many irrigation practices (Munns and Tester, 2008; Rengasamy, 2010). With the exception of some C4 photosynthetic plants for which sodium is an essential micronutrient, most crop plants are natrophobic (Ohnishi *et al.,* 1990). Salt stress effects on plant growth include nutritional constraints by decreased uptake of phosphorus, nitrate and calcium, ion toxicity mainly due to Na^+, Cl^- and $SO4^{2-}$ and osmotic stress leading to turgor loss and cell volume change (Chinnusamy and Zhu, 2003). In addition to

slower growth rates, salinity also causes a reduced tillering and abnormal reproductive development, with obvious profound and negative impact on agricultural yield (Chinnusamy and Zhu, 2003).

1.4.1. Plant responses to salinity

Plants respond to stress as individual cells and also synergistically as a whole organism. The plant responses to salinity comprise two phases, an "osmotic phase" and an "ionic phase". At the cellular level, salt stress affects ion and osmotic homeostasis. Sodium (Na^+) and chloride (Cl^-) are the two key ions responsible for both ion-specific and osmotic damage. These ions interfere with the non-covalent interactions between amino acids in the proteins, leading to protein conformational changes and loss of function, disruption of protein synthesis and interference with enzyme activity (Bhandal and Malik, 1988; Blaha *et al.,* 2000; Chinnusamy and Zhu, 2003). Another important cause of damage are the reactive oxygen species (ROS) generated by salt stress (Miller *et al.,* 2010). Changes in the plasma membrane electrical potential are also a direct consequence of ion imbalance caused by salt stress (Serrano and Rodriguez-Navarro, 2001). Additionally, osmotic imbalances lead to turgescence loss with consequent cell volume changes and retraction of the plasma membrane from the cell wall. Physiologically, the osmotic phase occurs immediately after salt application and consists of stomatal closure, with consequent increase of leaf temperature and decrease of photosynthetic activity (Passioura and Munns, 2000; Sirault *et al.,* 2009), as well as inhibition of cell expansion and cell division (Yeo *et al.,* 1991; Fricke, 2002; Munns and Tester, 2008). The "ionic phase" consists in the accumulation of salt over time, resulting in premature senescence of older leaves and in toxicity symptoms such as chlorosis and necrosis in mature leaves (Munns, 2002; Tester and Davenport, 2003; Munns *et al.,* 2006).

1.4.2. Transducing stress signals

Several chemicals are not only essential for plant growth and development but also play important roles in integrating various stress signals and in controlling downstream stress responses. These roles include the control of gene expression and the regulation of various transporters/pumps, among other biochemical reactions. The primary salt stress signals (ionic and osmotic stress) are transduced through specific signalling pathways, namely through Ca^{2+} and receptor kinase pathways. Changes in membrane polarization caused by the entrance of Na^{+} into the cells activate Ca^{2+} channels (Ca^{2+} ATPases and H^{+}/Ca^{2+} antiporters). These Ca^{2+} waves form rapidly, within 5-10 seconds of salt stress, propagate quickly, and are thought to be one of the earliest events in salt signalling (Lynch *et al.,* 1989). Therefore, cytosolic Ca^{2+} oscillation acts as a second messenger in salt stress (Sanders *et al.,* 1999; Knight and Knight, 2000). Additionally, several Ca^{2+}-binding proteins play important roles in stress tolerance. For example, the calcium-dependent protein kinases (CDPKs) mediate cellular responses either directly by changing enzymatic activities through protein phosphorylation, or indirectly by changing gene expression patterns (Sathyanarayanan and Poovaiah, 2004), particularly of transcription factors (Mehlmer *et al.,* 2010). Calmodulins (from CALcium MODULating proteIN) and Calcineurin B-like proteins (CBLs) are other important calcium sensors that, although missing enzymatic activity, can bind to target proteins and regulate their activity (Ranty *et al.,* 2006). Salt stress-induced Ca^{2+} signals can also be perceived by the SOS pathway, which is of vital importance for ion homeostasis regulation under salinity. SOS3 (salt overly sensitive 3) binds Ca^{2+} and activates de SOS2 kinase, which in turn phosphorylates the SOS1 Na^{+}/H^{+} antiporter that pumps Na^{+} out of the cytosol. The SOS3-SOS2 kinase complex also regulates Na^{+} compartmentation by activating NHX1 and by restricting Na^{+} entrance into

the cytosol by inhibiting the plasma membrane Na^+ transporter HKT1 (Chinnusamy and Zhu, 2003).

The retraction of the plasma membrane caused by Na^+ entry into cells activates several membrane-bound proteins, including receptor kinases. The first osmosensor kinase identified in plants was the histidine kinase ATHK1, from Arabidopsis (Urao *et al.,* 1999), which was confirmed to act as an osmosensor to transmit the stress signal to a downstream MAPK cascade (Tran *et al.,* 2007; Wohlbach *et al.,* 2008). The ligands and the downstream signalling molecules sensed by receptor kinases are not yet fully known. Extracellular signals, such as hormones, small peptides, small chemical molecules and physical stimuli are probable ligands. The downstream intracellular events possibly include kinase cascades (e.g. MAPK), Ca^{2+} ions, ROS signalling, metabolic adjustments, and membrane dynamics (Osakabe *et al.,* 2013). Other signalling molecules are synthesized in response to salt stress, likely playing important roles on plant salt tolerance, namely phosphoinositides (Parre *et al.,* 2007; Tang *et al.,* 2007), Reactive Oxygen Species (Kim *et al.,* 2010; Ruan *et al.,* 2011; Deng *et al.,* 2014, Hoang *et al.,* 2015), Nitric Oxide (Zhang *et al.,* 2007a) and sugars (Kempa *et al.,* 2007). The phytohormones which are essential for plant growth and development also play an important role in integrating various stress signals and controlling downstream stress responses. For example, abscisic acid (ABA) is involved in regulation of plant water balance and osmotic stress tolerance (Zhu, 2002), jasmonic acid (JA) is implicated in salt stress adaptation in rice, barley, grapevine and wheat (Moons *et al.,* 1997; Walia *et al.,* 2007; Ismail *et al.,* 2011; Qio *et al.,* 2014, respectively) while salicylic acid (SA) is involved in the oxidative stress responses triggered by NaCl and osmotic stress (Borsani *et al.,* 2001; Jayakannan *et al.,* 2015).

1.4.3. Salinity tolerance

The major goal of salinity tolerance research is to generate plants capable of maintaining growth and productivity in saline soils. However, the success of breeding programs aimed to produce salt-tolerant crops is limited by the absence of a clear understanding of the molecular basis of the tolerance mechanisms, as well as by their multigenic nature, which impairs traditional breeding techniques such as introgression (Dewey, 1962; Flowers and Yeo, 1995; Roy *et al.,* 2014). For plant salt tolerance, there are three important conditions: (1) to prevent or alleviate damage (i.e., detoxification), (2) to re-establish homeostasis in the stressful conditions, and (3) to resume plant growth even if at lower rate. The operating mechanisms that plants have to successfully manage the salt stress include:

i) **Ion exclusion** - consisting of Na^+ and Cl^- transport in roots in order to reduce the accumulation of these ions at toxic levels in leaves. Ion exclusion involves the coordinated action of several transporters at the plasma membrane and tonoplast (Tester and Davenport, 2003; Plett and Moller, 2010).

(ii) **Tissue tolerance** - where high Na^+ concentrations are found in leaves but compartmentalized at the cellular and intracellular level, especially in the vacuole (Tester and Davenport, 2003). In order to maintain equal osmotic potentials in vacuole and cytoplasm, solutes not harmful to cellular biochemistry must accumulate in the cytoplasm. These compatible solutes or “osmoprotectants” include secondary metabolites such as quaternary ammonium compounds (e.g. glycinebetaine), polyols (e.g. mannitol) and core metabolites like proline and sucrose (Rathinasabapathi, 2000). The production of enzymes catalyzing ROS detoxification is also important to achieve tissue tolerance (Roy *et al.,* 2014).

iii) **Osmotic tolerance** – known to be regulated by long-distance signals triggered before shoot Na^+ accumulation. Such mechanisms are pretty much unknown, but must involve rapid and long-distance signalling, probably via

processes such as ROS waves (Mittler *et al.,* 2011; Suzuki *et al.,* 2012), Ca^{2+} waves (Choi *et al.,* 2014), or even long-distance electrical signalling (Maischak *et al.,* 2010).

1.4.4. Susceptibility to salt stress: contribution of the epigenetic background

Epigenetics contributes enormously to the control of gene expression associated with developmental or environmental clues. Epigenetic inheritance, being a source of interesting polymorphisms able to generate useful variation for selecting superior genotypes, has proven to be a very promising field for future studies on plant stress resistance (Springer, 2013). Moreover, epigenetics could also contribute to natural variation within species and even to local adaptation. For example, the contrasting morphological differences of mangrove plants (*Laguncularia racemosa*) grown on riverside or on salt marsh is accompanied by a considerable hypermethylation of riverside plants as compared with those of salt marsh plants (Lira-Medeiros *et al.,* 2010). In *Cannabis sativa*, varieties with contrasting cold acclimation capacities showed distinctive changes in their chromatin state. In particular, the *C. sativa* varieties that acclimated more efficiently showed increased methylation levels at COR gene *loci* when deacclimated, suggesting a link between *locus* specific methylation and deacclimation (Mayer *et al.,* 2015). Specific epigenetic backgrounds associated with distinct varieties are particularly evident in rice. Genome-wide studies by MSAP analysis revealed a differential methylation between contrasting rice genotypes differing in their salt-responsive characteristics (Karan *et al.,* 2012) and drought resistance (Wang *et al.,* 2011). More recently, it was shown that salt-tolerant and salt-sensitive rice varieties display differential methylome flexibility under salt stress, with the tolerant rice varieties being able to adjust more rapidly their methylation levels under salt stress (Ferreira *et al.,* 2015) (*see* Chapter 2).

1.5. The model plant *Oryza sativa*

With a 491.4 million tonnes forecast for global production in 2016, rice is one of the most cultivated cereals in the world, only surpassed by maize, while from a cultural, economic and nutritional point of view, it is the most important crop worldwide (Khush, 1997; Bouman *et al.,* 2007; http://www.fao.org/worldfoodsituation/csdb/en/). Unlike the other major cereals, more than 90% of the global rice is consumed by humans. Given the predicted rise in the world's population, it is likely that rice consumption, and therefore rice demand, will increase over the next decades. However, despite being the staple food for more than half of the planet's population, a large part of rice production is still lost due to adverse environmental conditions. Therefore, active research on stress tolerance mechanisms in rice has turned this crop one of the most studied plant species nowadays. Rice is considered the model plant for cereals (Coudert *et al.,* 2010), mainly due to the high synteny with other cereal species (Moore *et al.,* 1995), its relatively small genome size (321Mb) (Kawahara *et al.,* 2013) and the availability of a high quality genome annotation (International Rice Genome Sequencing Project, 2005). Moreover, rice has been claimed as an epigenetic model, thanks to the growing collection of T-DNA mutants targeting epigenetic regulators (epimutants) allowing functional studies on gene regulation (Krishnan *et al.,* 2009). Besides helping to understand the role of epigenetics on phenotype variation, the epimutant plants provide great experimental systems to directly address the function of cytosine methylation without the problems found in using methylation inhibitors (epi-drugs), e.g. 5-azacytidine or zebularine (Baubec *et al.,* 2009). Opposite to animals, plants can tolerate epimutations quite well, although aberrant morphological phenotypes are frequently obtained (Kakutani *et al.,* 1996). Mutant analysis of regulatory components involved in methylation/demethylation pathways provides valuable insights about the functional mechanisms of the several epigenetic processes. Inclusively,

there are available data sets of several plant methylomes which can be used to, for instance, select differential epigenetic regions as possible targets for genetic manipulation.

1.6. Thesis objectives and outline

The innovation of DNA sequencing technology in the past years enabled a huge progress in genome biology. However, DNA sequence information alone is not enough to understand how the genome works. A high organization and a tight control of DNA accessibility allowing the expression of a set of genes in space and time are therefore crucial. Several players and mechanisms are already known to be involved in the regulation of chromatin states affecting transcription, such as DNA methylation, histone modifications, histone variants, nucleosome positioning, etc. The study on such regulation that does not directly involve DNA sequence is called “epigenetics”. Epigenetic regulation plays a very important role in a wide range of biological phenomena, including abiotic stress responses, although the precise mechanisms are yet poorly understood.

The main goal of this thesis was to assess how different aspects of epigenetic regulation, namely DNA methylation and histone modifications, are involved in salt stress plant responses. Rice was chosen as model, not only for being a very important crop but also for its high sensitivity to salt stress, although the degree of tolerance is known to vary among rice genotypes.

Our first hypothesis was that DNA methylation patterns are different in contrasting rice genotypes regarding its salinity tolerance. To test this hypothesis we used an ELISA-based technique, quite innovative in plants, to determine the relative global DNA methylation levels. In Chapter 2 we analysed the differences in global DNA methylation levels among different rice genotypes and described how they may account for the rice salt

tolerance. We also described the substantial differences in DNA methylation levels between roots and leaves.

Having also found distinct patterns of DNA methylation when comparing susceptible *versus* tolerant rice varieties, in Chapter 3 we examined in more detail the methylome dynamics induced by salt stress in the salt-tolerant rice variety Pokkali. We were mainly interested in identifying differentially methylated regions between the control and the salt stress samples, and in understanding the putative role of those regions as gene expression regulators.

Histone modifications are another epigenetic event with important regulatory impact on gene expression. The Chapter 4 was dedicated to the analysis of histone modifications landscape controlling the activation of a salt-responsive gene, *OsRMC* (rice *ROOT MEANDER CURLING*) that previous genotyping studies (by EcoTILLING) demonstrated that is highly conserved in rice varieties (Negrão *et al.,* 2013). We detected a differential enrichment of euchromatic marks upon salt stress depending on the promoter region, suggesting a nucleosome repositioning underlying *OsRMC* activation by salt stress.

Finally, Chapter 5 presents a thorough discussion of the results described in this thesis, and of the need for higher resolution techniques in the epigenetic field due to tissue-specific epigenetic regulation.

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Chapter 2

Salt tolerant and sensitive rice varieties display differential methylome flexibility under salt stress

Liliana J. Ferreira contributed to the planning and execution of all experiments presented in this Chapter, as well as to the discussion, interpretation and preparation of the manuscript. The statistical analysis was developed with the collaboration of João Maroco and the phenotypic assays had the collaboration of Vanessa Azevedo.

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Chapter 2:
Salt tolerant and sensitive rice varieties display differential methylome flexibility under salt stress

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2.1. Abstract

DNA methylation has been referred as an important player in plant genomic responses to environmental stresses but correlations between the methylome plasticity and specific traits of interest are still far from being understood. In this study, we inspected global DNA methylation levels in salt tolerant and sensitive rice varieties upon salt stress imposition. Global DNA methylation was quantified using the 5-methylcytosine (5-mC) antibody and an ELISA-based technique, which is an affordable and quite pioneer assay in plants, and *in situ* imaging of methylation sites in interphase nuclei of tissue sections. Variations of global DNA methylation levels in response to salt stress were tissue- and genotype-dependent. We show a connection between a higher ability of DNA methylation adjustment levels and salt stress tolerance. The salt-tolerant rice variety Pokkali was remarkable in its ability to quickly relax DNA methylation in response to salt stress. In spite of the same tendency for reduction of global methylation under salinity, in the salt-sensitive rice variety IR29 such reduction was not statistically supported. In 'Pokkali', the salt stress-induced demethylation may be linked to active demethylation due to increased expression of DNA demethylases under salt stress. In 'IR29', the induction of both DNA demethylases and methyltransferases may explain the lower plasticity of DNA methylation. We further show that mutations for epigenetic regulators affected specific phenotypic parameters related to salinity tolerance, such as the root length and biomass. This work emphasizes the role of differential methylome flexibility between salt tolerant and salt sensitive rice varieties as an

important player in salt stress tolerance, reinforcing the need to better understand the connection between epigenetic networks and plant responses to environmental stresses.

2.2. Introduction

Soil salinity is a major environmental constraint to crop production with negative impacts on growth rates, tillering and seed production (Munns and Tester, 2008). Rice (*Oryza sativa L*.) is the world's most important food crop facing particular problems with cultivation under adverse climate conditions. Due to the large genetic variability, rice varieties possess different degrees of salt sensitivity (Negrão *et al.,* 2011). Salinity tolerance is a quantitative trait controlled by multiple genes (Chinnusamy *et al.,* 2005) which can be involved in signal transduction, ion transportation, metabolic pathways and transcription regulation. Epigenetic mechanisms such as DNA methylation, histone modifications, nucleosome positioning and small non-coding RNAs, act in coordination to influence chromatin structure and gene expression (Henderson and Jacobsen, 2007; Chinnusamy and Zhu, 2009; Kim *et al.,* 2010; Santos *et al.,* 2011a). DNA methylation levels are modulated by an intricate interplay of DNA methyltransferases, DNA demethylases and other mechanisms, such as RNA-directed DNA methylation (RdDM) pathway mediated by siRNAs, but little is known regarding the mechanistic process of establishing, maintaining and removing methylation marks (Laird, 2010). Also, the functional significance of DNA methylation in plant response to environmental stress conditions is still largely unknown. Variations of epigenetic patterns and chromatin structure in relation to environmental conditions have been inspected by molecular and cytological approaches. Fluorescence *In Situ* Hybridization (FISH) enabled to detect major reorganization of highly condensed heterochromatic domains as ribosomal chromatin after imposing salt stress or hypomethylating agents e.g. 5-azacytidine (5-AC) (Santos *et al.,* 2011b). The methylation-sensitive

amplified polymorphism (MSAP) technique has been used to study the impact of stress on global DNA methylation (Wang *et al.,* 2011a; Wang *et al.,* 2011b; Karan *et al.,* 2012; Fulnecek and Kovarik, 2014).

This work focused in evaluating global DNA methylation levels in rice varieties with contrasting behaviors in response to salt stress. Shifts on global DNA methylation were detected after salt stress imposition. In addition, these shifts were influenced by genotype and tissue specificity. While the salt tolerant rice variety Pokkali was able to rapidly reduce DNA methylation under salt stress, the salt sensitive variety IR29 did not show such methylome flexibility, suggesting a link between the plasticity of DNA methylation and plant performance under salt stress. Furthermore, mutations of epigenetic modulators affected specific phenotypic parameters related to salinity tolerance such as root length and biomass.

2.3. Material and methods

2.3.1. Plant material, growth conditions and salt stress treatments

Salt tolerant and sensitive rice varieties (*Oryza sativa L.*) were provided by the International Rice Gene Bank held at the International Rice Research Institute (IRRI), Philippines. The ‘IR29’ is an *indica* variety referred as salt-sensitive standard (Bonilla *et al.,* 2002). ‘Pokkali’ is also an *indica* variety and has been widely used as salt-tolerant donor in breeding programs (Zeng, 2005). The ‘FL478’, also known as IR66946-3R-178-1-1, is a salt tolerant recombinant inbred line developed at IRRI and was used because their parents are ‘Pokkali’ and ‘IR29’ (Walia *et al.,* 2005). The *japonica* variety Nipponbare has been described as salt susceptible (Karan *et al.,* 2012).

Rice mutants for epigenetic regulators were originally developed by Gynheung An (Kyung Hee University, Crop Biotech Institute, Korea). In this study, the T-DNA insertion lines, 4A-01884 and 3A-08043 carrying mutations for a histone acetyltransferase and a DNA methyltransferase, respectively

were used (Jeon *et al.,* 2000; Jeong *et al.,* 2006). The Dongjin variety described as relatively salt tolerant (Sohn *et al.,* 2005) was also used since it is the wild-type from which these mutants originated.

Rice seeds were surface sterilized in 0.1% Benlate solution for 30 min at 50ºC, rinsed with sterile water, soaked in 70% ethanol for 1 min and washed with a solution of 2% sodium hypochlorite containing 0.02% Tween 20 for 30 min. After successive washings in sterile water, the seeds were placed on filter paper soaked in water and allowed to germinate for 3 days at 28ºC. Germinated seedlings were transferred to a hydroponic system containing Yoshida's medium (Yoshida *et al.,* 1976). Plants were allowed to grow in a growth-chamber at 28ºC/24ºC in a 12h photoperiod regime (500 µEm-2s-1) and with 70% humidity. The salt stress was imposed on 14-days-old rice seedlings by supplementing Yoshida's medium with 200 mM NaCl (EC=24 dS/m; EC = electrical conductivity). Root and leaf tissues were collected separately after 1 and 24h of salt treatment, frozen in liquid nitrogen and kept at -80ºC.

Seeds from rice mutants were heat-treated for 5 days in a convection oven set at 50ºC to break seed dormancy, then placed on filter paper soaked in water and incubated at 28ºC in the dark for 48h to germinate. The experimental design was a split plot design with 5 lines per tray and 26 plants per genotype. Two pre-germinated seeds were sown per hole on a styrofoam seedling float device and the emerging radicle was carefully inserted through the nylon mesh. The styrofoam device was suspended on a tray filled with only distilled water, since the endosperm has enough nutrients for the seedlings to grow normally for 3-4 days. After this period, trays were filled with Yoshida solution (Yoshida *et al.,* 1976). Two distinct salinity assays were performed. In "Assay 1", 4-days-old seedlings were subjected to an initial salinization of EC = 6 dS/m during one week that was then increased to EC = 12 dS/m for another week. In "Assay 2", the salt imposition was applied in 12-days-old seedlings, with an initial salinization of

EC = 6 dS/m for 3 days followed by a week in EC = 12 dS/m. In the control trays no salt was added. The solutions were renewed every 8 days and the pH daily checked and maintained at 5.0. The experiment was conducted in the growth chamber as mentioned above. All trays had two check entries: the susceptible 'IR29' and the tolerant 'Pokkali'.

2.3.2. Quantification of global DNA methylation

A relative quantification of global DNA methylation levels was obtained with an ELISA-based colorimetric assay using a commercially available kit, the MDQ1 (Imprint® Methylated DNA Quantification kit, Sigma Aldrich). The procedure was according to manufacturer's instructions. Even though some differences in methylation can occur between replicates, the use of multiple biological and technical replicates allowed a consistent calculation of the relative amounts of DNA methylation. Genomic DNA of roots and leaves from rice lines was isolated by using the DNeasy® Plant mini kit (Qiagen). Three independent DNA extractions were performed from a pool of twelve 14-days-old rice seedlings subjected to 1 or 24h of salt treatment. One hundred nanograms of genomic DNA per sample was immobilized on strip wells with high affinity for DNA and incubated at 60ºC. The methylated DNA was detected using optimized antibody and reagents with high specificity to 5-mC and then quantified colorimetrically. The absorbance was read on a microplate reader at 450 nm (Biotek Power Wave XS). The percentage of DNA methylation was calculated relative to an internal standard methylated control DNA supplied in the kit and according to the manufacturer's protocol. The following steps were followed: (a) average the A450 replicates for the blank, samples and methylated control DNA and (b) use the formula [(A450 av sample - A450 av blank)/(A450 av methylated control DNA - A450 av blank)] x 100 for calculation of % methylation of the samples relative to the methylated control DNA.

2.3.3. Imaging of 5-methylcytosine in interphase nuclei of tissue sections

Root-tips of 14-days-old seedlings were excised and fixed in 4% (w/v) formaldehyde freshly prepared from paraformaldehyde in PEM buffer (50 mM PIPES; 5 mM EGTA; 5 mM MgSO4; KOH pH 6.9) for 1h at room temperature and then washed in TBS for 10 min. Root tips were sectioned using a Vibratome Series 1000 (TAAB Laboratories Equipment Ltd, Aldermarston, UK) and allowed to dry on multi-well slides (Menzel-Glaser). Root sections of approximately 15-20 µm thickness containing about two cell layers showed good tissue preservation and integrity. The slides were pre-treated by washing in 3% Decon90 (detergent) for at least 1h, thoroughly rinsed with distilled water and coated with a freshly prepared solution of 2% (v/v) 3-aminopropyltriethoxysilane (APTES, Sigma) in acetone for 10 s and activated with 2.5% (v/v) glutaraldehyde (Sigma) in PBS for 30 min, rinsed in distilled water and air-dried. Prior to immunofluorescence, tissue sections were dehydrated in an ethanol series and digested with an enzyme mixture of cellulase 1.5% (w/v) (Onozuka R-10, Japan) and pectolyase 0.5% (w/v) (Sigma) in EB (0.4 mM citric acid; 0.6 mM trisodium citrate, pH 4.8) for 1h at room temperature. After washing 10 min with PBS, the sections were permeabilized with 0.1% Triton X-100 (v/v) in PBS for 3 min, washed again 10 min in PBS and dehydrated in an ethanol series and air-dried.

For *in situ* detection of 5-mC, a mouse monoclonal antibody against 5-methylcytosine (1:100, Santa Cruz Biotechnology) was used, followed by a secondary antibody (goat anti-mouse Alexa Fluor 488 1:500, Invitrogen). After blocking with 1% (w/v) Bovine Serum Albumine (Roche) dissolved in PBS/0.1% Triton X-100 for 1h, the tissue sections were incubated with the primary antibody overnight at 4ºC. After washes in PBS/0.1% Triton X-100, incubation with the secondary antibody was performed at 37ºC for 90 min. The nuclei were counterstained with DRAQ5 (1:1000 in PBS, Cell Signaling Technology) for 10 min, rinsed briefly in PBS and mounted in Vectashield

antifade solution (Vector Laboratories). Confocal optical section stacks (z-step size of 1 µm) were collected with a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg GmbH). The microscopy data were transferred to Image J and processed using constant parameters to minimize operator errors. The fluorescence intensity was measured in Z-projections of consecutive optical confocal sections using the average intensity parameter for each stack individually. The arbitrary units correspond to the values of the Raw Integrated Density measurement. Finally, Adobe Photoshop 5.0 (Adobe systems Inc., Mountain View, CA) was used for image composition.

2.3.4. Expression studies of DNA methyltransferases and demethylases by real-time quantitative PCR (qPCR)

Total RNA from roots and leaves of 'Pokkali' and 'IR29' was isolated from a pool of twelve 14-days-old rice seedlings subjected to either 1 or 24h of salt imposition (200 mM NaCl). The RNA extraction procedure followed the manufacturer's instructions (Zymo Resarch). The isolated total RNA was treated with TURBO DNA-free (Ambion) to eliminate any possible DNA trace. The cDNA first strand was synthesized from 4 µg of total RNA using the Randon Hexamer primer and according to the instructions from the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Real-time quantitative PCR was performed using the LightCycler 480 system (Roche) and the SYBR Green I Master mix (Roche). The PCR running conditions were as follows: one cycle at 95ºC for 5 min and 45 cycles of amplification at 95ºC for 10 s, 60ºC for 10 s and 72ºC for 10 s. All qPCR experiments were performed on two biological replicates and the CT values were calculated from means of three technical replicates. The relative quantification of gene expression was calculated with kinetic PCR efficiency correction using the comparative Ct method (2(-ΔΔCt) to determine the relative expression of transcripts relative to an endogenous control. The rice gene Ubiquitin-conjugating Enzyme E2 (*OsUBC2*, LOC_Os02g42314) was used as

endogenous gene, due to its stability under salt stress, to normalize the relative expression of the target transcripts (Figure S4). The qPCR reactions were performed with specific primers for the DNA demethylases DNG701 and DNG710, and for the DNA methyltransferase OsDRM2, listed on Table S1.

2.3.5. DNA methylation analysis by McrBC digestion

Genomic DNA from 'Pokkali' and 'IR29' leaves was isolated as referred above. Genomic DNA (1.5 µg) was digested overnight at 37ºC with 25 units of McrBC enzyme (New England Biolabs) in a final volume of 50 µl following the manufacturer's instructions. Digested and negative control samples were subjected to PCR amplification to detect the methylation status of eight selected transposable elements (TE-I_Os04g19320, TE-I_Os04g17620, TE-II_Os04g087100, Chr3-AnacA2_TE, Chr8-Tnr8_TE, Chr9-Ty3/gypsy_TE, Chr12-centromeric-like_LTR, Tos17), one repetitive sequence (Telomere_repetitive seq), four selected salt-stress responsive genes (*OsRMC*, *OsHKT5*; *OsSalT*, *OsNHX1*) and two constitutively expressed genes (eEF, OsActin). The primers were designed with NCBI software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/). The sequences are indicated in the table S2.

2.3.6. Genotyping rice T-DNA insertion lines

T-DNA insertion lines (4A-01884 and 3A-08043) carrying mutations for a histone acetyltransferase and a DNA methyltransferase, respectively, were PCR-genotyped to confirm the T-DNA insertion, using the flanking primer sets indicated in Table S3. According to the supplier, in the line 3A-08043, the insertion site is located in the sixth exon of *OsDRM2* (LOC_Os03g02010) and in the line 4A-01884, in the fourth exon of *OsHAC704* (LOC_Os06g49130) (Figure S1). Total RNA extracted from the

homozygous plants was used to confirm the silencing of *OsDRM2* (coding for a DNA methyltransferase) and *OsHAC704* (coding for a histone acetyltransferase) genes through semi-quantitative RT-PCR (Figure S2).

2.3.7. Phenotypic evaluation of rice plants with mutations for epigenetic regulators

At panicle emergence, at least 10 randomly selected panicles were harvested and the number of filled and empty grains was recorded. Spikelet fertility were estimated as the ratio of number of filled grains to total number of reproductive sites (florets) and expressed as percentage. The progeny of homozygous mutant plants was evaluated for salinity tolerance at the seedling stage as previously described. The Modified Standard Evaluation System (SES) for rice was used to rate the visual symptoms of salt injury as described by (Gregorio *et al.,* 1997). The shoot length (cm) was measured from the base of the stem to the tip of the topmost leaf of the plants. Root length (cm) was measured for each plant. Fresh weight (FW) of shoots and roots were determined immediately after collecting the samples, followed by tissue drying at 50ºC for 7 days for the determination of dry weight (DW). Water content was estimated as follows %WC = (FW-DW)/DW)*100 and biomass refers to the dry weight. All parameters are presented as percentage relative to control plants.

2.3.8. Statistical data analysis

All results were statistically analyzed with one-way ANOVA after checking for assumptions (normality with the Shapiro-Wilk test and Homoscedasticity with the Leven test). Where the ANOVA revealed statistically significant effects, the Tukey-HSD test was also used to identify the groups where the values obtained differed. All statistical analysis was

conducted with SPSS Statistics (v. 21, SPSS An IBM Company, Chicago, IL). Statistical significance was assumed for $p<0.05$.

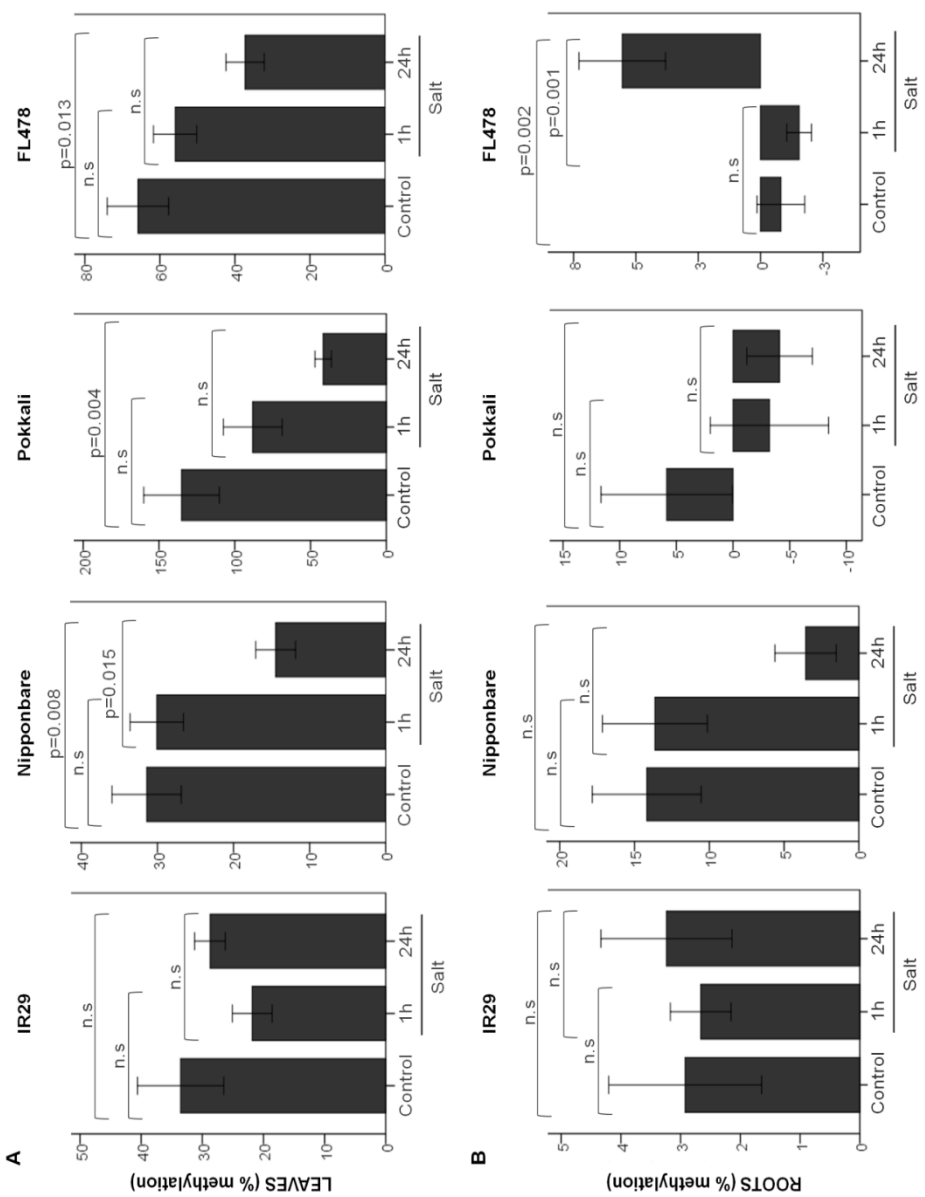
2.4. Results

2.4.1. Salt stress induced DNA demethylation

The quantification of global amount of 5-mC was performed in root and leaf tissues of distinct rice varieties under salt stress to assess tissue and genotype specificity. A common observation to all rice varieties analyzed is the differential methylation levels between roots and leaves. Global DNA methylation levels are lower in roots than in leaf tissues (Figure 1A,B).

In leaves, a short exposure to salt stress (1h) was sufficient to cause a decrease of DNA methylation, although not statistically significant which continued to decline upon a more extended stress imposition (24h) except for 'IR29' (Figure 1A).

Figure 1: Global DNA methylation levels in salt tolerant and sensitive rice varieties. Genomic DNA from leaves and roots of 'IR29', 'Nipponbare', 'Pokkali' and 'FL478' in control and salt stress conditions (1h and 24h of 200 mM NaCl) were used to determine the relative global DNA methylation using a commercial ELISA-based kit. The methylation values represented in the plots correspond to percent methylation of the samples relative to a methylated control DNA supplied with the kit. (A) In leaves, there were statistically significant differences between control and 24h of salt stress in the Nipponbare, Pokkali and FL478 varieties ($F(2,24)=6.68$: $p=0.005$; $F(2,24)=6.39$: $p=0.006$; $F(2,24)=4.993$: $p=0.015$, respectively). (B) For the methylation % in the roots of Nipponbare at 24h of salt stress imposition, there was a significant effect of the treatment on the roots percentage methylation ($F(2,24)=3.601$; $p=0.043$), although the Tukey's HSD test only revealed marginally significant differences between the control and 24h treatments ($p=0.06$). In 'FL478' the methylation % was higher in 24h of salt stress than in control ($F(2,24)=10.946$; $p<0.001$). Statistical significance was assumed for $p<0.05$. The "p" value was calculated according to the Tukey HSD. N.S: Not Statistically Significant.


A
B
IR29
Nipponbare
Pokkali
FL478
LEAVES (% methylation)
ROOTS (% methylation)
n.s
p=0.008
p=0.015
p=0.004
p=0.013
p=0.002
p=0.001
Control
1h
24h
Salt

The salt tolerant ‘Pokkali’ showed a remarkable ability to alter DNA methylation levels with a 70% decline of total DNA methylation upon salt stress. In ‘Nipponbare’ and ‘FL478’ the methylation loss was about 54% and 43%, respectively. In contrast, in the salt susceptible ‘IR29’, the methylation loss under salinity was only 14%, with no-statistical significance (Figure 1A).

In roots, the effect of salt stress on global DNA methylation was not such conspicuous, with exception for ‘FL478’, which showed statistically significant changes in global DNA methylation in response to 24h of salt stress imposition (Figure 1B).

The visualization of DNA methylation *loci* at single interphase nuclei of root tissues though immunofluorescence with a specific antibody against 5-mC was achieved in root sections of ‘Pokkali’ and ‘IR29’ with 14-days-old seedlings in control and stress conditions (24h NaCl). The 5-mC *loci* distribution pattern in rice interphase nuclei consisted of widespread bright fluorescent spots indicating methylation (Figure 2A-L). An important observation is that salt stress triggered a reorganization of spatial patterns of methylation *loci*. In addition, the fluorescence intensity was measured and is presented in Arbitrary Units (AU). No statistical significant interaction was found between variety and salt treatment ($F(1,381)=2.219$; $p=0.137$), meaning that IR29 and Pokkali varieties responded in a similar way to salt stress (Figure 2N).

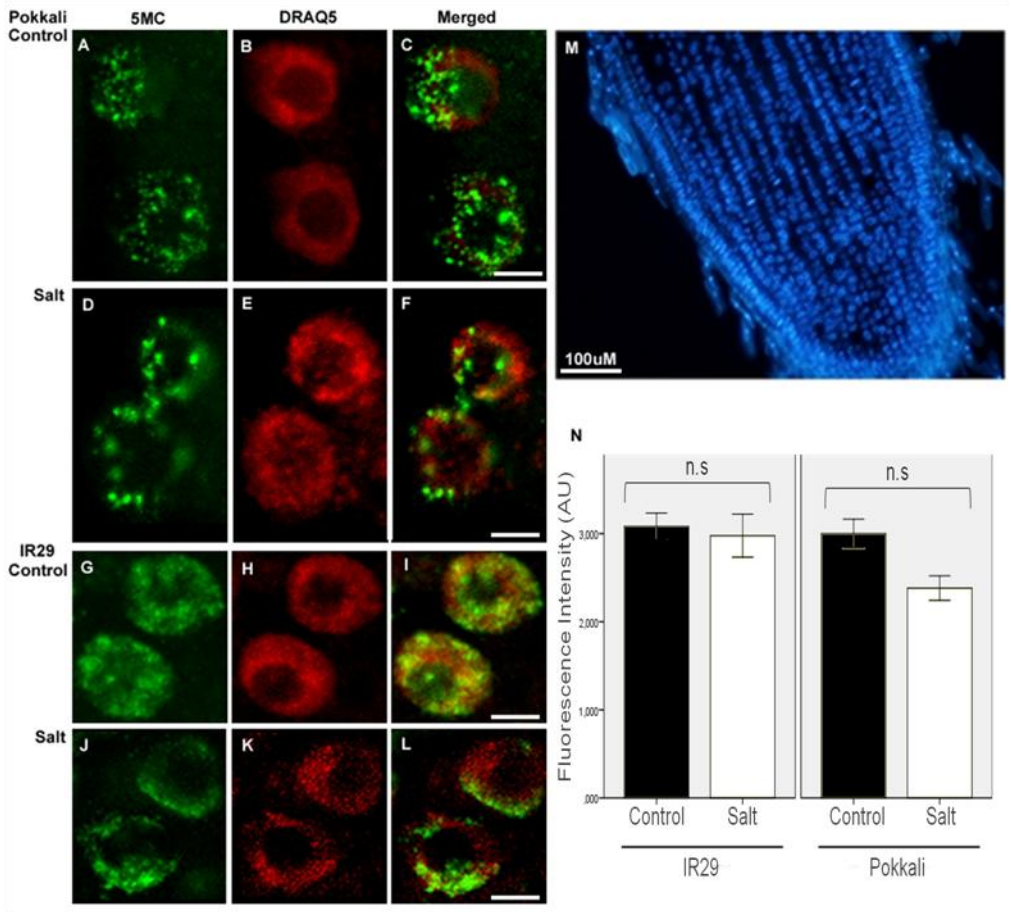


Figure 2: 3D imaging of DNA methylation in single interphase nuclei. Immunofluorescence with a specific antibody against 5-methylcytosine was performed in rice root sections of 'Pokkali' (A-F) and 'IR29' (G-L) 14-days-old seedlings in control conditions or after 24h of exposure to salinity stress (200 mM NaCl). Bar = 10 µM. (M) Rice root-tip section obtained with a vibratome, and stained with DAPI. (N) Fluorescence intensity was calculated using Image J. The values obtained are shown as arbitrary units (AU).

2.4.2. Salt-stress effects on DNA methyltransferases and demethylases expression patterns were genotype specific

To better understand DNA methylation dynamics under salt stress, the expression patterns of a rice DNA methyltransferase (MTase) and two DNA demethylases were studied in leaf and root tissues from 'Pokkali' and 'IR29'

in control conditions or after salt stress imposition. The OsDRM2 is a DNA methyltransferase required for *de novo* methylation (encoded by LOC_Os03g02010), while the DNG701 (encoded by LOC_Os05g37350) and DNG710 (encoded by LOC_Os05g50290) act as putative DNA glycosylases/lyases involved in targeted removal of 5-mC from methylated DNA (Ortega-Galisteo *et al.,* 2008; La *et al.,* 2011).

In leaves, the *DNG701* expression pattern was basically similar in both varieties with a decrease after 1h of salt stress and an increase after a more prolonged exposure to salt (24h) (Figue 3A,D). Regarding the *DNG710*, a gradual increase in expression was detected in leaves of both varieties along salt stress imposition (Figure 3B,E). Concerning the methyltransferase (*OsDRM2*), the most striking observation is the distinct expression profiles of 'Pokkali' and 'IR29' under prolonged exposure to salt. The *OsDRM2* expression level is increased in 'IR29' but not in 'Pokkali' (Figure 3C,F). For 'IR29', a statistically significant interaction exists between treatment and gene expression ($F(4,18)=41.3$; $p<0.001$), as well as between treatments (control and the two stress periods) ($F(2,18)=56.601$; $p<0.001$). Similarly, in 'Pokkali' a statistically significant interaction was found between gene expression and treatment ($F(4,18) =10.647$; $p<0.001$) or between treatments ($F(2,18)=23.085$; $p<0.001$).

In what concerns to root tissues, a differential expression profile of demethylases was detected between the two varieties under analysis. In 'Pokkali', salt stress induced the expression of demethylases (Figure 3A,B) while in 'IR29' the DNG701 showed declining expression (Figure 3D). Regarding the methyltransferase, a significant repression was detected after 1h of salt stress (Figure 3C,F). A more prolonged exposure to salt (24h) was enough to recover the expression of demethylases and methyltransferase to the original levels

Statistical data analysis revealed that 'IR29' showed a significant interaction between gene and treatment ($F(4,18)=13.367$; $p<0.001$) but the

differences between treatments were not significant (F(2,18)=0.739; p=0.492). 'Pokkali' showed a statistically significant interaction between genes and treatment (F(2,18)=8.397; p=0.001), as well as between treatments (F(2,18)=8.397; p<0.001).

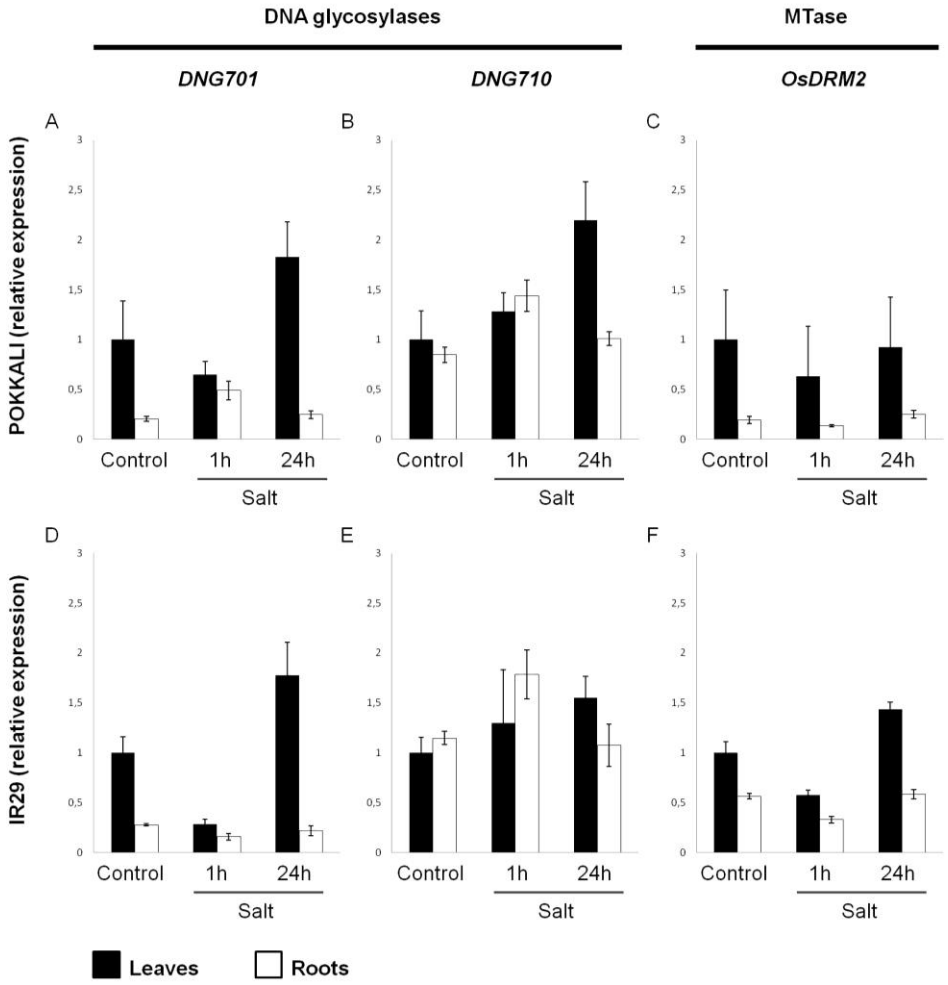


Figure 3: DNA demethylases (*DNG701* and *DNG710*) and DNA methyltransferase (*OsDRM2*) expression studies. Quantitative real-time PCR reactions were performed with cDNA prepared from total RNA extracted from leaves and roots of 14-days-old seedlings of 'Pokkali' (A-C) and 'IR29' (D-F) subjected to salinity for 1h or 24h (200 mM NaCl). The data were normalized to the internal control Ubiquitin-conjugating Enzyme E2 (OsUBC2). The mean expression value of control was normalized to 1 and the other means values represent fold change in expression. Error bars represent standard deviation.

2.4.3. DNA methylation of stress related targets

To evaluate whether salt stress-induced demethylation was preferentially occurring at transposable elements (TEs), repetitive sequences or specific salt-stress related genes, a McrBC digestion followed by methylation-sensitive PCR (MS-PCR) was performed in Pokkali and IR29 varieties. The McrBC is an endonuclease that binds the methylated half-sites (G/A)mC and cleaves between them (Lippman *et al.,* 2004). Successful amplification after digestion indicates lack of methylation. In control conditions, McrBC digestion detected methylation of a Ty3-gypsy TE located on chromosome 9 of "Pokkali" only and a telomeric repetitive sequence in 'Pokkali' and 'IR29' (Figure 4A). While in 'Pokkali' both sequences suffered demethylation under salt stress, in 'IR29' the methylation status of the telomeric repetitive sequence was not altered by salt stress (Figure 4A). In addition, the LTR sequence located on Chr12 failed to amplify in control and stress conditions in 'IR29' while in 'Pokkali' that occurred only under salt stress conditions. Regarding specific salt-stress related genes, the McrBC digestion did not indicate methylation in control or salt stress conditions (Figure 4B).

2.4.4. Mutations of epigenetic modulators affected phenotypic parameters related to salinity tolerance

To investigate a putative connection between chromatin remodeling enzymes and phenotype under salt stress, specific T-DNA insertion lines were submitted to genotyping. The following homozygous lines were selected, one encoding a histone acetyltransferase (*oshac704*) and other encoding a DNA methyltransferase (*osdrm2*). The *osdrm2* mutant plants (but not the *oshac704*) showed impaired seed germination (roughly only 50% of the seeds were viable, data not shown). Additionally, the *osdrm2* seeds

required more than 3 days after imbibition for coleoptile emergence while the WT (Dongjin) seeds only required 36 to 48h.

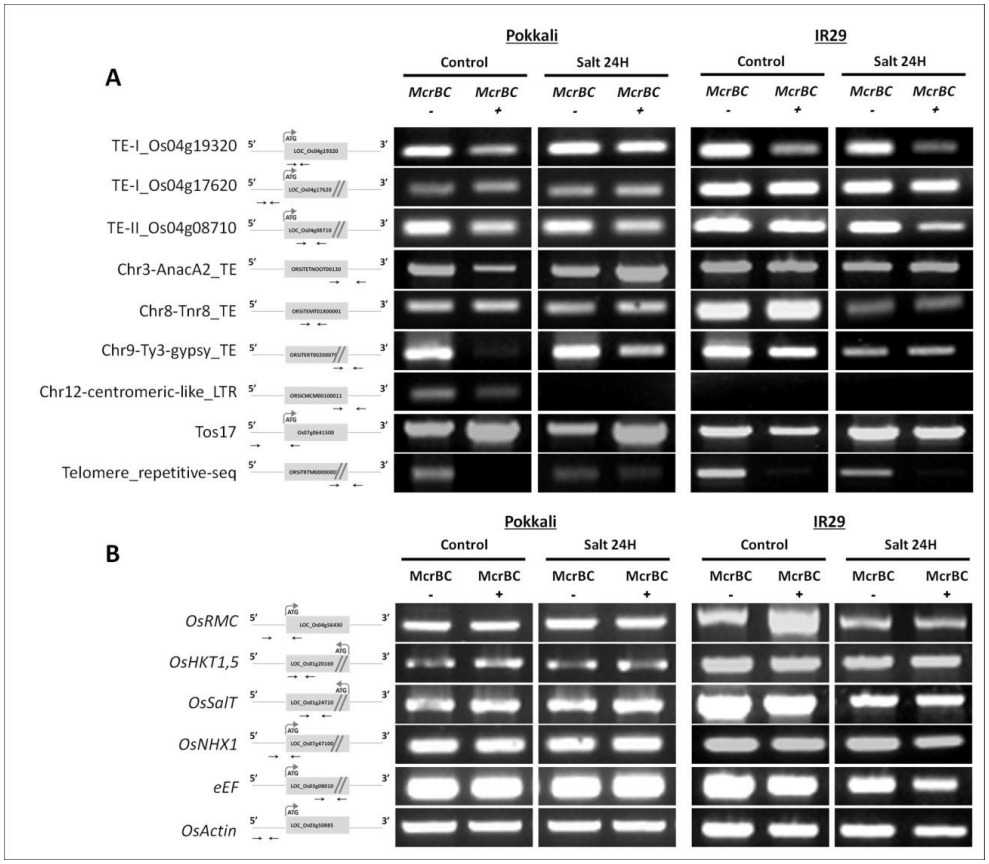


Figure 4: McrBC based methylation analysis. Leaves of Pokkali and IR29 rice varieties in control and salt stress conditions (24h of 200 mM NaCl) were used. (A) Transposable elements (TEs) and repetitive sequences (B) Genes involved in rice responses to salt stress and constitutively expressed genes. The position of primers for all selected TE elements and stress related genes is indicated.

The negative effect in seed germination affected subsequent growth, particularly root length and biomass (Table 1). In contrast, the differences between *oshac704* and WT plants were mostly observed at the shoot level, with mutant plants showing a higher shoot development (Table 1). Both *oshac704* and *osdrm2* mutants had reduced spikelet fertility about half of that observed for WT plants grown under normal conditions as shown in Figure S3.

Table 1: Phenotypic evaluation of rice mutants

	"Assay 1"						"Assay 2"					
	Control			Salt stress			Control			Salt stress		
	WT	*osdr m2*	*osha c704*	WT	*osdr m2*	*osha c704*	WT	*osdr m2*	*osha c704*	WT	*osdr m2*	*osha c704*
S.E.S	2	2	2	6	8	7	1	2	2	6	5	5
Shoot length (cm)	35.1 ± 1.74	32.94 ± 5.70	39.7 ± 2.58	19.35 ± 2.37	16.10 ± 2.33	16.00 ± 1.76	43.35 ± 2.48	41.70 ± 6.03	45.61 ± 4.08	35.40 ± 2.30	32.59 ± 2.54	35.70 ± 4.23
Shoot FW (g)	0.69 ± 0.05	0.62 ± 0.12	0.76 ± 0.13	0.14 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	0.76 ± 0.05	0.48 ± 0.11	0.68 ± 0.07	0.32 ± 0.03	0.20 ± 0.03	0.34 ± 0.05
Shoot DW (g)	0.11 ± 0.02	0.11 ± 0.05	0.11 ± 0.02	0.04 ± 0.003	0.03 ± 0.004	0.03 ± 0.003	0.10 ± 0.01	0.06 ± 0.02	0.09 ± 0.02	0.05 ± 0.005	0.05 ± 0.01	0.05 ± 0.01
Shoot Water Content (%)	84.34	81.77	85.84	73.99	72.45	74.63	86.84	87.02	86.69	84.93	83.05	85.46
Root length (cm)	13.55 ± 0.06	12.50 ± 1.27	12.95 ± 0.90	13.25 ± 2.10	12.45 ± 1.48	8.05 ± 1.55	17.03 ± 1.52	14.87 ± 2.50	16.08 ± 1.97	11.93 ± 0.91	11.28 ± 1.71	11.60 ± 1.61
Roots FW (g)	0.38 ± 0.033	0.31 ± 0.055	0.36 ± 0.065	0.103 ± 0.005	0.085 ± 0.015	0.075 ± 0.012	0.43 ± 0.03	0.28 ± 0.07	0.32 ± 0.06	0.23 ± 0.03	0.17 ± 0.01	0.20 ± 0.04
Root DW (g)	0.035 ± 0.003	0.023 ± 0.02	0.039 ± 0.01	0.01 ± 0.0004	0.01 ± 0.002	0.01 ± 0.004	0.06 ± 0.05	0.02 ± 0.005	0.03 ± 0.01	0.02 ± 0.002	0.02 ± 0.001	0.02 ± 0.004
Root Water Content (%)	90.81	92.64	89.11	86.55	87.04	84.86	85.48	91.64	90.88	90.11	90.18	90.11

Lower levels of DNA methylation were observed in the *oshac704* mutant in control conditions as compared to WT ($p<0.05$) (Figure 5). There were no significant interactions between mutants/WT and treatment ($F(2,18)=.285$; $p=0.755$) meaning that the treatment effect was not different for the mutants and WT and consisted on DNA demethylation under salt stress. For example, regarding the *hac704* mutant, it has undoubtedly less methylation than the WT, losing 32% of methylation with salt stress. The methylation in control is 67.6% (relative value) representing the absolute methylation that the mutant actually has. Under salt stress, the methylation is 45.6% (relative value) and thus, for *oshac704*, the loss of methylation under salt stress was about 32%. Interestingly, the salt-stress related demethylation registered in the mutants (34% and 32% for *osdrm2* and *oshac704,* respectively) was more extensive than in the WT (25%) (Figure 5).

For the functional characterization of *osdrm2* and *oshac704* mutants, the salinity tolerance was evaluated at the seedling stage as described in the material and methods. Two salt stress assays, differing on the plant developmental stage at which salinization is imposed and duration of salt stress period, were performed as described in methods section. The modified standard evaluation score was used for rating the visual symptoms of salt toxicity (Gregorio *et al.,* 1997). At the end of ”Assay 1” all plants showed a complete growth cessation with most leaves drying, and thus were classified as susceptible. In comparison to mutants the WT showed slightly lower symptoms of salt injury (Table 1, Figure 6A). In “Assay 2”, when salinization was imposed to 12 days-old seedlings, growth retardation was observed but most leaves maintained the green color with rolled tips. Compared to WT, the mutants showed a slightly better performance under stress conditions as long as the stress was imposed to 12 days-old seedlings (Table 1, Figure 6B). A slightly enhanced performance of epi-mutants may well be linked to their higher flexibility in changing DNA methylation levels under stress.

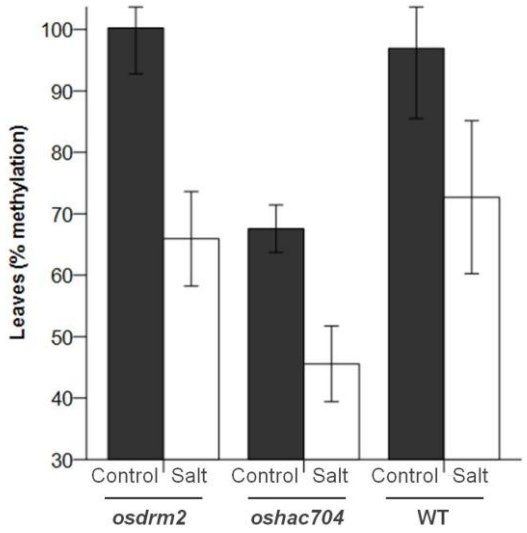


Figure 5: Global DNA methylation levels in rice mutants. Leaves of the *osdrm2* and *oshac704* rice mutants and WT (Dongjin) in control or salt stress conditions (24h of 200 mM NaCl) were used. The methylation values represented in the plots correspond to percent methylation of the samples relative to a methylated control DNA supplied with the kit. There were statistically significant differences between varieties ($F(2, 18)=6.628$; $p=0.007$) as well as treatment ($F(1,8)=14.307$; $p=0.001$). The control samples always had higher % of methylation than the salt stress ones. The *oshac704* mutant had the lowest methylation percentage ($p<0.05$).

Concerning the salt effects on growth, in both assays the mutants showed a marked reduction of shoot length when compared to WT. The root length was similarly affected by salinity in WT and mutants in “Assay 2”, but in “Assay 1” the *oshac704* mutant exhibited a severe growth reduction as compared to WT and *osdrm2* mutant (Figure 6C). The percentage of water content was not substantially different between WT and mutants in both assays (Figure 6D). In “Assay 1”, the mutants showed reduced biomass as compared to WT (except for roots of *osdrm2*), while in “Assay 2” the opposite results were observed (Figure 6E). Statistical analysis was performed by one-way ANOVA to calculate the effects of the treatment (control *versus* salt imposition) and the mutations [WT (Dongjin) *versus*

mutants]. Statistical significance was assumed for $p<0.05$. The detailed statistical analysis of Figure 6C-E is presented in Table S4.

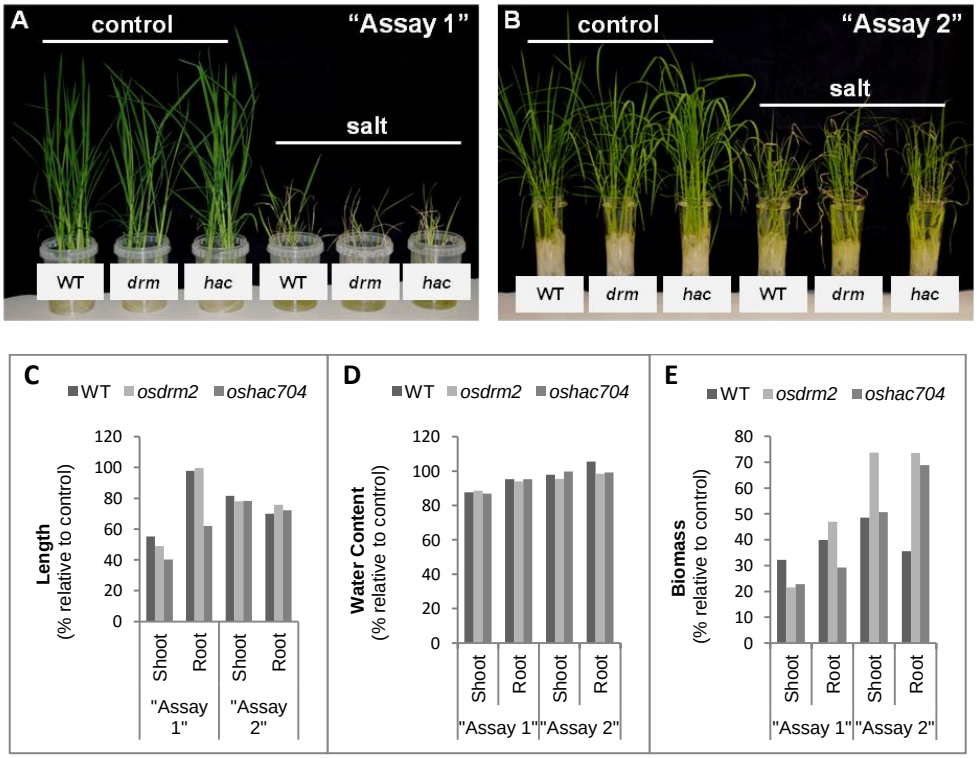


Figure 6: Phenotypic evaluation of epigenetic rice mutants under salt stress. The assays "1" and "2" (**A** and **B**, respectively) refer to the application of salt stress at distinct developmental stages for different time periods. The length and biomass values correspond to a % of change in salt relative to control. For details, see description in material and methods. **(C)** Shoot and root length of WT, *osdrm2* and *oshac704* in salt stress relative to control conditions. **(D)** Percentage of water content in shoots and roots of WT, *osdrm2* and *oshac704* in salt stress relative to control conditions. **(E)** Biomass of shoots and roots of WT, *osdrm2* and *oshac704* in salt stress relative to control conditions.

2.5. Discussion

In this study, we evaluated global DNA methylation levels in distinct tissues of salt tolerant and sensitive rice varieties upon salt stress imposition. Global DNA methylation levels were quantified by the ELISA-based technique assay. This experimental approach has been widely used to measure DNA methylation in cancer research because it is relatively inexpensive and enables a fast, reliable and accurate processing of a high amount of samples (Kinnally *et al.,* 2011). The quantification of global DNA methylation levels by an ELISA assay is still quite new in plants and so far, it was only used to quantify DNA methylation of cork oak genome (Ramos *et al.,* 2013). In the present work, shifts in global DNA methylation levels were detected after salt stress imposition. In addition, these shifts were influenced by genotype and tissue type. The salt tolerant rice variety Pokkali was able to rapidly reduce DNA methylation under salt stress while the salt-sensitive 'IR29' showed a low ability to adjust DNA methylation levels upon salt stress suggesting a link between the plasticity of DNA methylation and plant performance under salt stress.

Previous studies in rice using the MSAP technique reported DNA demethylation events upon salt stress imposition (Wang *et al.,* 2011b; Karan *et al.,* 2012). Also, in rapeseed, salt-induced demethylation assessed by MSAP markers was much stronger in the tolerant variety Exagone as compared with the sensitive 'Toccata' (Marconi *et al.,* 2013). Our cytological analyses revealed a spatial reorganization of DNA methylation patterns in response to salt stress which is consistent with the reorganization of heterochromatic domains in rice interphase nuclei after salt stress imposition or treatment with the 5-azacytidine (5-AC) hypomethylating drug (Santos *et al.,* 2011b).

DNA methylation levels were significantly lower in root tissues than in leaves. Tissue-dependent DNA methylation patterns have also been previously reported but a convincing explanation for those variations is still

missing (Lu *et al.,* 2008; Wang *et al.,* 2011a; Karan *et al.,* 2012). Some authors have argued that tissue-specific biological functions should imply a tissue-specific gene regulation, eventually involving differential DNA methylation (Aceituno *et al.,* 2008). Comparative transcription profiling under salinity stress showed that shoots and roots operate differently between rice varieties. For example, shoots of susceptible varieties have a higher number of salinity-induced transcripts than those of tolerant varieties (Walia *et al.,* 2005), opposite to what was observed for roots of the same varieties ('FL478' and 'IR29') (Cotsaftis *et al.,* 2011). Therefore, a tissue-dependent DNA methylation pattern may not exclusively explain tissue-specific gene regulation. Another possible explanation for tissue-dependent DNA methylation pattern relates to tissue complexity and differentiation. The presence of undifferentiated meristematic tissue in roots could also explain the lower methylation levels observed in this tissue as compared to leaves, since meristematic root tip cells tend to have more open chromatin states and less DNA methylation (Lafon-Placette *et al.,* 2013).

Demethylation events occurred mainly on TE-related *loci* and these genetic elements comprise approximately 30% of the rice genome (http://rice.plantbiology.msu.edu/). More interestingly, a LTR located in the centromeric region of chromosome 12 (Chr12-centromere-like LTR) failed to be amplified in 'Pokkali' under salt stress conditions which may suggest a salt-induced transposition event. It is well known that stress can cause widespread genomic restructuring events including transposition of mobile elements (Madlung and Comai, 2004and in rice, mainly *in vitro* cell or tissue culture have been associated with transposition (Hirochika *et al.,* 1996; Jiang *et al.,* 2003; Komatsu *et al.,* 2003; Kikuchi *et al.,* 2003). In addition, this LTR was undetectable in the IR29 variety, which could be related to modified transposition sites. Different transposition sites between varieties have been reported in other plant species, namely in Arabidopsis, where significant

transposition events were detected between 'Col' and 'Ler' ecotypes (Vaughn *et al.,* 2007).

It is not clear whether changes in DNA methylation are simple indirect effects of salt stress or a mechanism for regulation the expression of salt stress responsive genes. The production of reactive oxygen species (ROS) has been associated with stress (Hernández *et al.,* 1993; Hernández *et al.,* 1995; Zhu, 2003) and may affect DNA methylation levels (Weitzman *et al.,* 1994). During carcinogenesis, the 8-hydroxyguanosine, a product of oxygen radical damage, is able to replace guanine leading to altered DNA methylation patterns (Cerda and Weitzman, 1997; Franco *et al.,* 2008). However, because DNA demethylation occurs as early as 1h after salt stress independently of DNA replication, chromatin remodeling enzymes should be involved, specifically the ones involved in active demethylation (Steward *et al.,* 2002). In fact, our results point to an active demethylation in the tolerant 'Pokkali', since we observed an induction of DNA demethylases by salt stress imposition. In contrast, the susceptible 'IR29' responded to salt stress with the transcriptional induction of both DNA demethylases and DNA methyltransferases, which could explain the non-significant alteration of global DNA methylation levels.

Rice plants with mutations for epigenetic modulators are ideal tools to investigate putative links between epigenetic marks and effects at phenotypic traits. The rice mutant line 3A-08043 has a silenced *OsDRM2* gene, an MTase of the Domains Rearranged Methyltransferases (DRM) family involved in *de novo* methylation in all sequence contexts (Finnegan *et al.,* 1996; Henikoff and Comai, 1998; Finnegan and Kovac, 2000; Cao *et al.,* 2000). Due to the *OsDRM2* gene silencing, it would be expected a reduction of global DNA methylation levels in this line when compared to WT. However, in this study, we detected similar methylation levels between the mutant and the WT which could be explained by the presence of other members of this MTase family in rice (Sharma *et al.,* 2009). Likewise, it was

previously shown that knockout or knockdown of *OsDNG701*, a rice DNA glycosylase responsible for DNA demethylation, did not cause global DNA hypermethylation but rather a *locus*-specific DNA hypermethylation, namely in Tos17 (La *et al.,* 2011). Also in Arabidopsis, mutations in the DNA demethylation pathway affected only specific genes (Choi *et al.,* 2002; Gong *et al.,* 2002; Penterman *et al.,* 2007; Zhu *et al.,* 2007). Together with our results, it may well be possible that the loss of function of some chromatin remodelling enzymes can affect the writing and/or erasing of epigenetic marks in a *locus*-specific manner instead of genome wide effects. Interestingly, the rice mutant for the histone acetyltransferase revealed significantly lower global methylation than WT, illustrating the crosstalk between distinct epigenetic marks.

Regarding the phenotype of mutant plants, despite the marked reduction in spikelet fertility and impaired seed germination and development (particularly evident in the *osdrm2* mutant) plants were able to generate progeny. Contrary to mammals, where mutations in DNA methyltransferases have lethal effects (Li *et al.,* 1992), in plants and fungi mutations in single DNA methyltransferases do not cause significant impacts in phenotype (Kouzminova and Selker, 2001; Kankel *et al.,* 2003; Saze *et al.,* 2003). However, when looking at specific phenotypic parameters, it was possible to detect that root length and biomass of the *osdrm2* mutant was less affected by salinity than the WT.

In summary, the tolerant rice variety Pokkali exhibited a higher capacity for changing DNA methylation levels in response to salt stress. In contrast, the salt sensitive IR29 variety was unable to adjust its methylation levels. These findings suggest different epigenetic regulatory networks between rice varieties and may account for the variability of salt stress response/tolerance observed in rice. Further studies are needed to better understand the regulation of epigenetic marks and their impact in salt stress adaptation.

2.6. Acknowledgments

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2.8. Supporting information

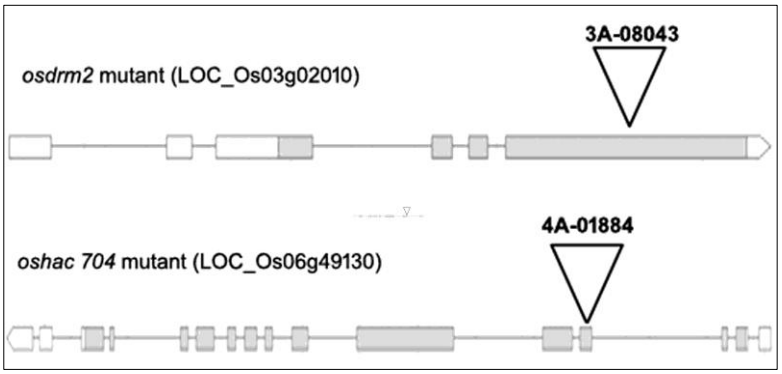

Figure S1: Schematic representation of the rice T-DNA insertion lines (http://signal.salk.edu/cgi-bin/RiceGE).

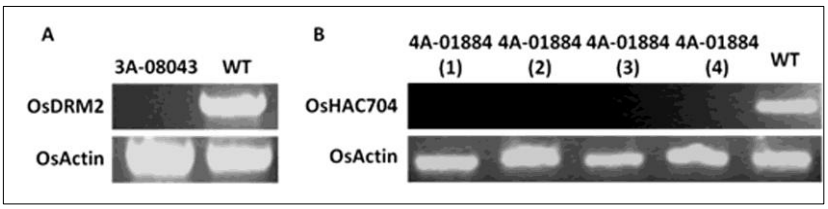

Figure S2: Expression studies of *OsDRM2* and *OsHAC704* in the T-DNA rice mutant lines.

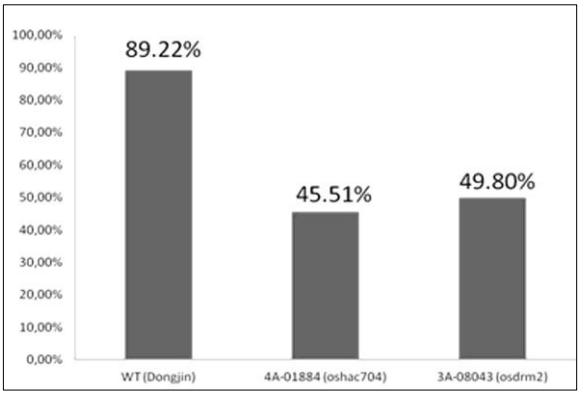

Figure S3: Spikelet fertility in WT (Dongjin) and T-DNA rice mutant lines.

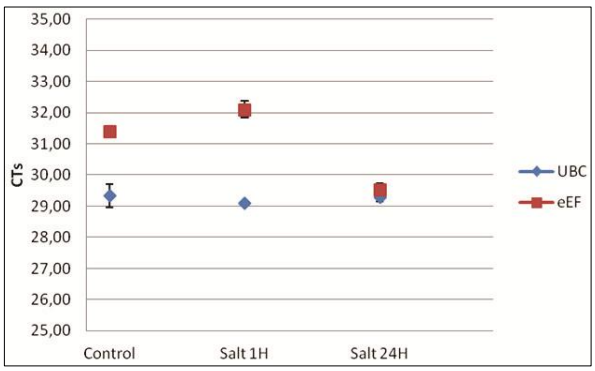

Figure S4: Threshold cycle (CTs) values for the ubiquitin-conjugating enzyme E2 (UBC2) and elongation factor (eEF) genes under salt stress conditions.

Table S1: List of Primers used for expression studies of DNA demethylases and DNA methyltransferase.

Genes	Primer forward	Primer reverse	References
DNG701	ATGGCGAAAGACGAGAACCC	TCGCACTGACCATGGGAAAG	This work
DNG710	CCTGCACTGACTGAACATGG	CTGGGAGCATCTGAAGAGGA	This work
OsDRM2	GTGGGCCATCTGGGAATCAA	CCTCTCATTGGGCAAGCTGA	This work
OsUBC2	TTGCATTCTCTATTCCTGAGCA	CAGGCAAATCTCACCTGTCTT	This work

Table S2: List of Primers used in the McrBC methylation analysis.

TEs / repetitive sequences	Primer forward	Primer reverse	References
TE-I_Os04g19320	GCCAGCTCCTCTCTCTCTCA	AGATACGGCTATTGGCGATG	[1]
TE-I_Os04g17620	AATCCTGAGCTTCCAGCAGA	TCAGGTGGAGTTTTGTGCAT	[1]
TE-II_Os04g08710	CCATCCTCTTACAGGGACCA	CCCGTCTTCCTCATTGATCT	[1]
Chr3-AnacA2_TE	TCTTCCTCCTCCCTCTCCTC	CTCGCGACTTCGATTAGACC	This work
Chr8-Tnr8_TE	TTGCAAAACAACAAAAATCTTGA	ACATTATTTCTGTAAATGGTGTATG	This work
Chr9-Ty3-gypsy_TE	TGTTATTTGYATTGATTTAATAGGG	ACTACCTCCCTCTCTCTCTCA	This work
Chr12-centromere-like_LTR	GTCACTCCCCGACCCTATCT	GCCGAGCTTCTTCTTGTCAC	This work
Tos17	GTCCCTATCCATGTGCTGGT	TGTTTACGCTCAGCAACACC	This work
Telomere_rep.seq	CCCCAACCTTAATGAAGCAA	CATCATAGCCCCCGTTGTTA	This work
Genes			
OsRMC	GAAGACCTGGTTCCGATTGA	CTGATGTTGGTGCTGCTCAT	This work
OsHKT1,5	TCCGACGTCCTAACCCTAGCTCTAC	TGGTGATGATTTACGCACATGAGAC	[2]
OsSalT	TTCCAGACCTTCCAAAGAATCCAA	ACAAGGAAATTTAAGCGACCACGA	[2]
OsNHX1	GCGGATCCACCTGGACTATC	GAAATCGGGATTTGGTATCGAG	[2]
eEF	ACCCTCCTCTTGGTCGTTTT	AAATACCCGCATTCCACAAC	[3]
OsActin	CCTCAGCCGCCTTTCACTAT	CATCTGTGGTGATGTGGCGA	This work

[1.] **Yin BL, Guo L, Zhang DF, Terzaghi W, Wang XF, Liu TT, *et al.*** (2008) Integration of cytological features with molecular and epigenetic properties of rice chromosome 4. *Mol. Plant* **1(5):** 816-829

[2.] **Negrão S, Courtois B, Ahmadi N, Abreu I, Saibo N, Oliveira MM** (2011) Recent updates on salinity stress in rice: from physiological to molecular responses. *Crit. Rev. Plant. Sci.* **30:** 329–377

[3.] **Figueiredo DD, Barros PM, Cordeiro AM, Serra TS, Lourenço T, Chander S *et al.*** (2012) Seven zinc-finger transcription factors are novel regulators of the stress responsive gene OsDREB1B. *J. Exp. Bot.* **63(10):** 3643-3656

Table S3: List of Primers used for genotyping rice T-DNA insertion lines.

T-DNA line	Genes	Primer forward	Primer reverse	References
3A-08043	*OsDRM2*	ACGCTTCAGTGGGTAACTGC	CTGCTGACATGGTAGCCTTT	This work
4A-01884	*OsHAC704*	GGCTCAACATCAGCAACGTC	TCCGATACACCATACGGGGA	This work

Table S4: Statistical analysis underlying phenotypic evaluation of rice mutants.

	Effects of the treatment Control *versus* Salt		**Effects of the mutations** WT (Dongjin) *versus* Mutants	
	"Assay 1"	**"Assay 2"**	**"Assay 1"**	**"Assay 2"**
Shoot length (cm)	F(1,52)=570.847 p<0.001	(F(1,104)=155.598 p<0.001	F(2,52)=5.717 p=0.006	F(2,104)=7.599 p<0.001
Root length (cm)	F(1,52)=21.68 p<0.001	F(1,52)=175.212 p<0.001	F(2,52)=22.669 p<0.001	F(2,52)=5.749 p<0.001
Shoot water content (%)	(F(1,52)=154.317 p<0.001	F(1,104)=36.495 p<0.001	F(2,52)=4.775 p=0.012	F(2,104)=9.799 p<0.001
Root water content (%)	F(1,52)=46.728 p<0.001	F(1,104)=0.907 p=0.343	F(2,52)=5.49 p=0.007	F(2,104)=5.59 p=0.005

Shoot biomass (g)	F(1, 52)=189.246 p<0.005	F(1,104)=303.536 p<0.001	p>0.05	F(2,104)=29.338 p<0.001
Root biomass (g)	F(1,52)=94.616 p<0.001	F(1,104)=22.76 p<0.001	F(2,52)=5.658 p<0.001	F(2,104)=13.656 p<0.001

Statistical analysis by one-way ANOVA to calculate the effects of the treatment (control versus salt imposition) and the mutations (WT (Dongjin) versus mutants). Statistically significance was assumed for $p<0.05$.

Chapter 3

Identification of Differentially Methylated Regions in a salt tolerant rice variety and their role in gene expression regulation

Liliana J. Ferreira contributed to the planning and execution of all experiments presented in this Chapter, as well as to the discussion, interpretation and preparation of the manuscript. The bioinformatic analysis was developed with the collaboration of Mark Donoghue, Filipe Borges and Rob Martienssen.

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Chapter 3:
Identification of Differentially Methylated Regions in a salt tolerant rice variety and their role in gene expression regulation

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3.1. Abstract

Rice is an important staple food crop for nearly half the world's population and is extremely sensitive to salt stress. Salt stress has been widely associated to changes in cytosine methylation levels. The functional role of DNA methylation in salt stress response/tolerance is still not well understood. In this study, we analysed the methylome dynamics in leaves of the salt tolerant rice variety Pokkali by high-throughput sequencing of immunoprecipitated anti-5-mC DNA (MeDIP-Seq). This strategy allowed the identification of 53 salt stress-specific Differentially Methylated Regions (sDMRs) between control and salt stress samples. In addition, a detailed analysis of these sDMRs revealed a loss of methylation in response to salt stress. The genome location of sDMRs, namely their proximity to genes, was, in some cases, correlated with salt stress gene induction, suggesting that they may act as gene expression regulators.

3.2. Introduction

Rice (*Oryza sativa L.*) is considered a model plant for cereals genomics since the order of genes on a chromosome is quite similar to that of other cereals including wheat and maize (Moore *et al.,* 1995). In addition, rice is one of the most important crops worldwide. Rice susceptibility to adverse environmental conditions causes serious problems to its cultivation in the current scenario of climate changes. Salt stress is a major threat to rice

productivity, thus there is an urgent need to gather new knowledge on salt tolerance mechanisms and on identifying salt tolerant genes in rice. The exposure to salt stress has been connected to chromatin remodelling, namely decondensation of heterochromatic domains such as ribosomal chromatin in rice interphase nuclei (Santos *et al.,* 2011). The functional role of DNA methylation in plant responses to stress is still largely unknown. In rice, changes in cytosine methylation levels in response to salt stress have been reported (Wang *et al.,* 2011; Karan *et al.,* 2012). We recently reported that rice varieties with distinct susceptibility to salt stress have different abilities to adjust global DNA methylation levels upon salt stress (Ferreira *et al.,* 2015). In response to salt stress, the tolerant rice variety Pokkali was more efficient in changing methylation levels than other varieties, suggesting a link between DNA methylation and salt tolerance.

Differentially methylated regions (DMRs) correspond to short genomic regions presenting contrasting methylation patterns between multiple sample contexts (Rakyan *et al.,* 2008). The DMRs have been mostly studied in humans and are currently regarded as functional regions possibly involved in gene transcriptional regulation (Wan *et al.,* 2015). In humans, the identification of DMRs have led to a classification according to distinct contexts including tissue-specific DMRs (tDMR), cancer-specific DMRs (cDMR), reprogramming-specific DMRs (rDMR), imprinting-specific DMRs (iDMR) and aging-specific DMRs (aDMR) (Doi *et al.,* 2009; Rakyan *et al.,* 2011; Zhang *et al.,* 2011; Heyn *et al.,* 2012; Bergman and Cedar, 2013; Slieker *et al.,* 2013; Smith and Meissner, 2013; Varley *et al.,* 2013; Wan *et al.,* 2015). In plants, DMRs have been also identified during *in vitro* dedifferentiation and regeneration of Populus (Vining *et al.,* 2013), between inbred lines of maize (Eichten *et al.,* 2013) and soybean (Schmitz *et al.,* 2013), in rice hybrids (Chodavarapu *et al.,* 2012) and between certain epialleles in Arabidopsis (Havecker *et al.,* 2012). Regarding abiotic stress

conditions, DMRs were identified in Arabidopsis after induced drought simulation (Colaneri and Jones, 2013).

The potential regulatory role of DMRs is still underexplored. The main objective of this study was to identify DMRs associated to salinity stress imposition. For that, we analysed the methylome dynamics in 'Pokkali' leaves by a genome-wide approach, namely the methylated DNA immunoprecipitation followed by high throughput sequencing (MeDIP-Seq). We report the identification of 53 DMRs between control and salt stress conditions. The detailed examination of these DMRs revealed the association of demethylation events with salt stress. Most DMRs were found in close proximity to specific genes, which may suggest a role in gene expression regulation. Therefore, the identification of DMRs in specific stress conditions (stress-specific DMRs, or sDMRs) may be an important tool to unveil hotspots of epigenetic regulation of salt stress responsive genes.

3.3. Material and methods

3.3.1. Plant material, growth conditions and salt stress treatment

The rice variety *Oryza sativa* ssp *indica* cv. Pokkali was used in this study. Rice seeds were surface disinfected with a benlate solution (0.1%) for 30 min at 50ºC, rinsed with sterile water, soaked in 70% ethanol for 1 min and washed with a solution of 2% sodium hypochlorite containing 0.02% Tween 20 for 30 min. After several washes with sterile water, seeds were germinated on Petri dishes containing 3MM paper embedded in sterile water, in the darkness, for 3 days, at 28ºC. Germinated seedlings were transferred to glass tubes containing Yoshida's medium (Yoshida *et al.,* 1976) and allowed to grow in a growth-chamber under 28ºC/24ºC in a 12h photoperiod regime 500 $\mu Em^{-2}s^{-1}$ and with 70% humidity. The salt stress treatment was applied to 14-days-old seedlings and consisted in supplementing the Yoshida's medium with 200 mM NaCl. Rice leaves were

collected after 24h of salt treatment, frozen in liquid nitrogen and kept at -80ºC.

3.3.2. Methylated DNA immunoprecipitation sequencing (MeDIP-Seq)

Genomic DNA from 'Pokkali' leaves was isolated using the DNeasy® Plant mini kit (Qiagen) according to manufacturer instructions. The DNA quality was evaluated through electrophoresis in agarose gel and through absorbance spectra using the Nanodrop. DNA was then sonicated to a length of approximately 150-400 bp and incubated using a monoclonal antibody highly specific to recognize 5-methylcytosine. The methylated enriched fraction of the immunoprecipitation DNA was then high throughput sequenced using the Illumina Hi-seq platform as a service provided by Active Motif. The immunoprecipitated DNA was amplified using barcoded Illumina primers to generate the final library for sequencing. A control input library was prepared by amplifying a small amount of DNA (pool of all samples) that did not go through the MeDIP step. To make the methylomes of our samples comparable and to ensure statistical strength, two biological replicates for each condition were used. In average, 15 million 50 bp single-ended reads were generated for each condition.

3.3.3. Mapping and processing the MeDIP-Seq reads

Rice (*Oryza sativa* ssp *japonica*, cv. Nipponbare) has a high quality reference genome sequence and gene annotation information (Kawahara, 2013) thus the reads were mapped to the Michigan State University Genome Annotation Project Database, version 6.1 (http://rice.plantbiology.msu.edu/). Raw reads were clipped and trimmed using Trimmomatic (Lohse *et al.,* 2012). The remaining high quality reads were mapped using GSNAP (Wu and Watanabe, 2005). Duplicate reads were removed with Samtools (public domain: http://samtools.sourceforge.net/; Li *et al.,* 2009).

3.3.4. Identification of Differentially Methylated Regions (DMRs)

Uniquely mapped reads were analyzed using the MEDIPS software package (Chavez *et al.,* 2010) to estimate methylation levels. Reads were extended by 250 bp. Differential methylation analysis was carried out using 100 bp windows across the genome (FDR < 0.1, log2FC(1.2), minimum mean counts per group = 2). Genomic regions that were statistically significantly differentially methylated between compared samples were considered to be differentially methylated regions (DMRs).

3.3.5. Bisulfite Sequencing (BS)

The BS method was used to validate the MeDIP-Seq data by tracking the methylation status of specific salt stress related DMRs. Five hundred nanograms of genomic DNA were subjected to bisulfite conversion using the EZ DNA methylation ^{TM} (Zymo Research) according to the manufacture's protocol. Four microliters of the bisulfite converted DNA was used for PCR amplification (Taq DNA polymerase from New England Biolabs) of selected regions, namely the DMRs 2 and 15 (see Supplementary Table S1 for primer sequences). The PCR product was cloned into the pCR™4-TOPO® Vector (Invitrogen Life Sciences Technologies) and used to transform *E. coli* DH5α competent cells. The plasmidic DNA was extracted and purified with the Easy spin plasmid DNA minipreps kit (Cytomed) and then about 20 clones were sent to sequence on Macrogen facilities (http://dna.macrogen.com/eng/). The Kismeth platform was used to design the primers for amplification of the bisulfite converted DNA and for sequencing analysis of the multiple clones (http://katahdin.mssm.edu/kismeth; Gruntman *et al.,* 2008).

3.3.6. Gene expression studies by quantitative real-time PCR

Total RNA from leaves of 'Pokkali' was isolated from a pool of twelve rice seedlings with 14-days-old subjected to 24h of salt stress (200 mM NaCl). The RNA extraction procedure followed the manufacturer's instructions (Zymo Resarch). The isolated total RNA was treated with TURBO DNA-free (Ambion) to eliminate any possible DNA trace. The RNA integrity was checked by agarose gel electrophoresis and RNA concentration and purity was measured with Nanodrop. The cDNA synthesis was performed with 4 µg of total RNA using the Randon Hexamer primer and according to the instructions from the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). The total cDNA obtained was diluted 5 times and 5 µl were used for PCR amplification. Real-time quantitative qPCR was performed using the LightCycler 480 system (Roche) and the SYBR Green I Master mix (Roche). The PCR running conditions were as follows: one cycle at 95ºC for 5 min and 45 cycles of amplification at 95ºC for 10 s, 60ºC for 10 s and 72ºC for 10 s. The CT values were calculated from means of three technical PCR replicates. The relative expression level of each transcript was calculated using the method "relative quantification with kinetic PCR efficiency correction". The rice gene Ubiquitin-conjugating Enzyme E2 (*OsUBC2,* LOC_Os02g42314) was used to normalize the relative expression of the target transcripts given its stability under salt stress (Ferreira *et al.,* 2015). All experiments were done with at least three biological replicates. Primers for genes located nearby some selected DMRs are listed in Supplementary Table S2.

3.3.7. Gene ontology analysis

The gene ontology (GO) classification was restricted to the set of genes under study and was based on information available on Rice Genome Annotation Project (http://rice.plantbiology.msu.edu/).

3.4. Results

3.4.1. The leaf methylome of the rice tolerant variety Pokkali

The methylome dynamics was studied in leaves of 14 days-old rice seedlings of the tolerant variety Pokkali in control or after salt-stress imposition. Two sequencing runs generated between 13.6 and 17.2 million 50SE raw sequencing reads for salt and control conditions, respectively. After removing the low-quality and clonal reads, 3.5 to 4.2 million uniquely mapped high-quality reads were retained for each of the four samples (Table 1). The methylome of 'Pokkali' consisted on an even distribution of DNA methylation throughout the entire chromosomes, without obvious enrichment on specific chromosome regions, such the pericentromeric heterochromatin (Figure 1A and S1).

3.4.2. Differentially Methylated Regions (DMRs) showed decreased methylation after salt stress

Differential methylation analysis was carried out on the MeDIP-Seq data using the MEDIPS program (see Methods). Fifty-three DMRs were detected between control and salt stress samples. DMRs within 500 bp from one another were merged to give a final total of 22 DMRs (ranging from 100 to 1000 bp) (Table 2). The distribution of DMRs along rice chromosomes is shown in Table 2. The higher number of DMRs was detected in chromosomes VI and XII (four and six DMRS, respectively) (Table 2). Crossing the MeDIP-Seq data with the rice genome annotation (http://rice.plantbiology.msu.edu/) and the RepeatMasker software (http://www.repeatmasker.org/), we further obtained a genomic landscape for all DMRs (Figure S2). For example, the DMR2 is located on chromosome I, upstream a chloride channel protein coding gene (LOC_Os01g65500) and a DNA binding protein coding gene (LOC_Os01g65490), indicating that one

A

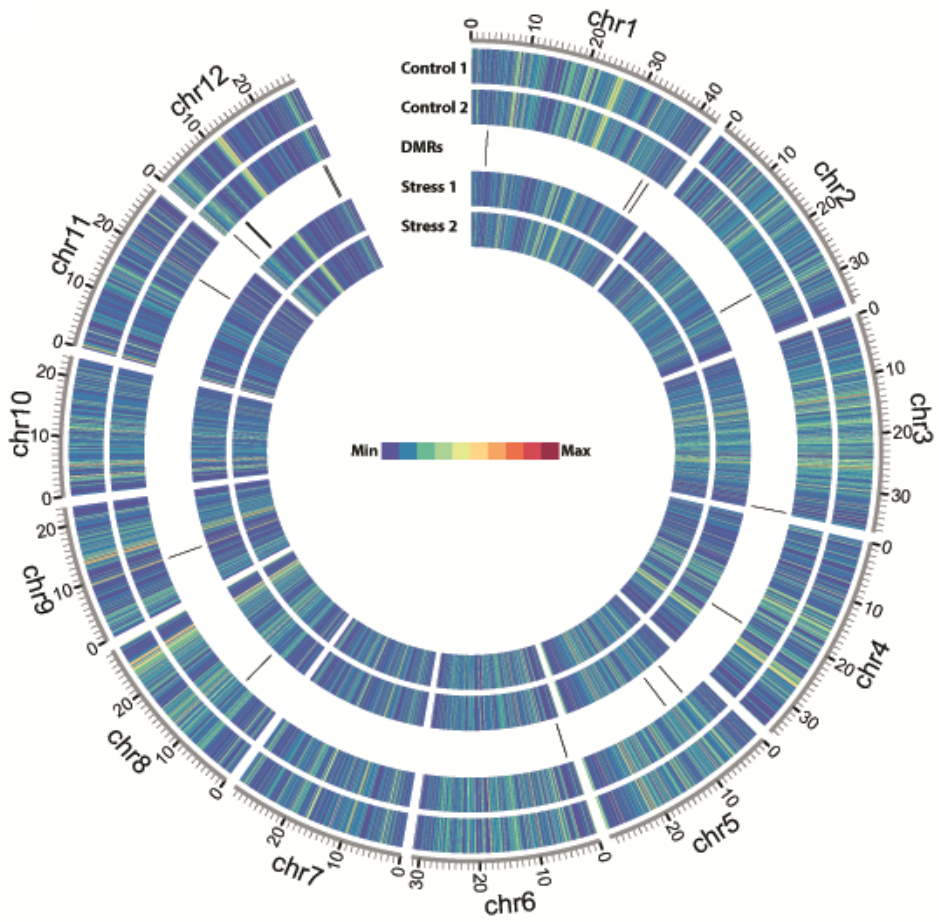
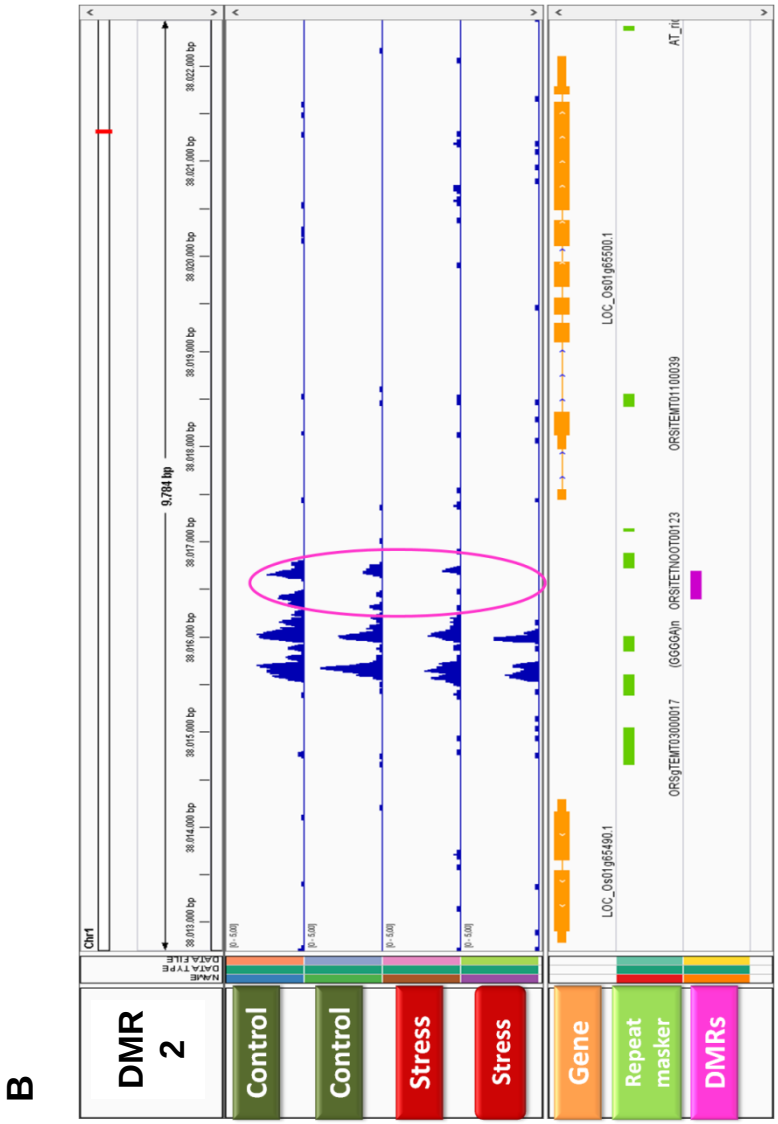


Figure 1: Identification of DMRs between control and salt stress samples in a salt tolerant rice variety. (A) A genome-wide view of DNA methylation of Pokkali leaves. The circos plot representation was used to show the location of Differentially Methylated Regions (DMRs) between control and salt stress conditions. The circos plot was based on the average RPM over 100000 bp windows. **(B)** An example of a DMR (DMR 2) exhibiting lower levels of DNA methylation in salt stress than in control conditions. The annotation of genes and repetitive sequences physically related to DMR is shown at the bottom. All the DMRs identified are shown in Figure S2.



DMR can potentially influence more than one gene (Figure 1B). The DMRs were analyzed according to its position relative to the nearest gene and more than 70% of the DMRs were located in close proximity to genes (less than 2 kbp away) (Figure 2A). Furthermore, over 75% of the DMRs identified were associated with transposable elements and repetitive sequences (Figure 2B).

Table 1: Summary of MeDIP-seq data analysis.

Condition	Biological replicates	Total reads	No. Uniquely mapped reads	Percent Uniquely mapped reads	Cytosine coverage % (Total C's = 63095915)						
					0x	1x	2x	3x	4x	5x	>5x
Control 1	2	17225011	4283278	24.87	82.82	8.05	2.48	1.37	0.92	0.67	3.70
Control 2	2	16081432	4075168	25.34	84.64	6.63	2.32	1.34	0.91	0.66	3.59
Stress 1	2	13681641	3639466	26.60	82.86	8.89	2.46	1.29	0.84	0.61	3.05
Stress 2	2	13845643	3562794	25.73	84.54	6.63	2.32	1.34	0.91	0.66	3.59
Input	1	14661478	7016939	47.86	51.2	22.17	14.59	7.39	3.03	1.06	0.57

Table 2: List of Differentially Methylated Regions (DMRs) between control and salt stress conditions. The position of DMRs at chromosome level is schematically represented. The genes adjacent to DMRs are also indicated.

Chr	DMR ID	Coordinat. start end	Repeat Masker annotation	Gene annotation	Position of DMR relative to the gene	Gene description
I 	1	3431001 3431100	AnacC1 transposon (ORSiTETN OOT00122)	LOC_Os01g 07270	78 bp downstream	Transposon
				LOC_Os01g 07280	506 bp downstream	Disease-resistance protein
	2	38016401 38016700	-	LOC_Os01g 65490	2100 bp ustream	DNA binding protein
				LOC_Os01g 65500	750 bp upstream	Chloride channel protein
	3	39466301 39466500	(CGG)n rich area	LOC_Os01g 67910	5' overlap	Expressed protein
				LOC_Os01g 67920	796 bp downstream	Tetratricopeptide repeat protein
II 	4	26500001 26500100	-	LOC_Os02g 43890	Within (intron/exon/intron)	Hypothetical protein
III 	5	36070201 36071200	AnacA2 transposon (ORSiTETN OOT00130)	LOC_Os03g 63840	4194 bp downstream	Expressed protein
				LOC_Os03g 63850	1972 bp upstream	OsFBDUF19 protein
IV 	6	22831201 22831400	(CGG)n rich area	LOC_Os04g 38390	780 bp downstream	Wound/stress protein
				LOC_Os04g 38400	2620 bp upstream	Ethylene-insensitive 3 protein
V 	7	4804401 4804700	AnacA10 transposon (ORSiTETN OOT00124)	LOC_Os05g 08760	Within (exon/intron)	Expressed protein
	8	4805301 4805500	-	LOC_Os05g 08760	Within (exon)	Expressed protein
	9	9320201 9320400	RIRE3 gypsy-type retrotranspo	LOC_Os05g 16420	1570 bp downstream	SHR5-receptor-like kinase protein

			son (ORSiTERTOOT00027)	LOC_Os05g16430	1300 bp upstream	SHR5-receptor-like kinase protein
VI 	10	962901 963200	E4 repeat sequence (ORSiOTOT00000050)	LOC_Os06g02680	680 bp upstream	Expressed protein
				LOC_Os06g02690	20 bp downstream	Expressed protein
	11	970501 970600	-	LOC_Os06g02700	Whitin (exon)	Retrotransposon Ty3-gypsy
	12	983401 983500	-	LOC_Os06g02730	3591 bp upstream	Aspartic proteinase nepenthesin-2 precursor protein
	13	1010401 1010700	(CGG)n rich area	LOC_Os06g02740	7261 bp upstream	Retrotransposon
				LOC_Os06g02770	Within (exon)	Expressed gene
VIII 	14	9021501 9021600	-	LOC_Os08g14950	1150 bp downstream	Receptor-like kinase 2 precursor protein
				LOC_Os08g14960	4240 bp upstream	Receptor-like kinase precursor protein
IX 	15	9475001 9475300	Ty3-gypsy retrotransposon (ORSiTERT00200079)	LOC_Os09g15470	3500 bp upstream	Retrotransposon Ty3-gypsy
				LOC_Os09g15480	1100 bp downstream	Ser/Thr-rich protein
XI 	16	20435601 20436000	-	LOC_Os11g34870	Within (intron)	Expressed protein
XII 	17	1446901 1447100	AnacA10 transposon (ORSiTETNOOT00124)	LOC_Os12g03601	519 bp upstream	Expressed protein
				LOC_Os12g03610	2283 bp upstream	Expressed protein
	18	4989301 4989600	noaCRR2 retrotransposon (ORSiTERTOOT00141)	LOC_Os12g09500	975 bp upstream	Cytochrome P450 protein
				LOC_Os12g09510	8570 bp upstream	Hypothetical protein
	19	5108601 5108800	Ty3-gypsy retrotransposon (ORSiTERT00200079)	LOC_Os12g09680	Within (intron)	Retrotransposon Ty3-gypsy

20	5301501 5301700	Centromere-like LTR transposon (ORSiCMC M00100011)	LOC_Os12g 10000	2500 bp upstream	Retrotransposon
			LOC_Os12g 10010	34 bp downstream	Expressed protein
21	25340601 25341000	(GGA)n rich area	LOC_Os12g 40930	155 bp upstream	Expressed protein
			LOC_Os12g 40940	4377 bp upstream	Expressed protein
22	25763601 2764100	noaCRR2 retrotransposon (ORSiTERTOOT00141)	LOC_Os12g 41630	4000 bp upstream	OsFBX463 – F-box domain protein
			LOC_Os12g 41634	Within (exon)	Expressed protein
			LOC_Os12g 41640	800 bp upstream	Expressed protein

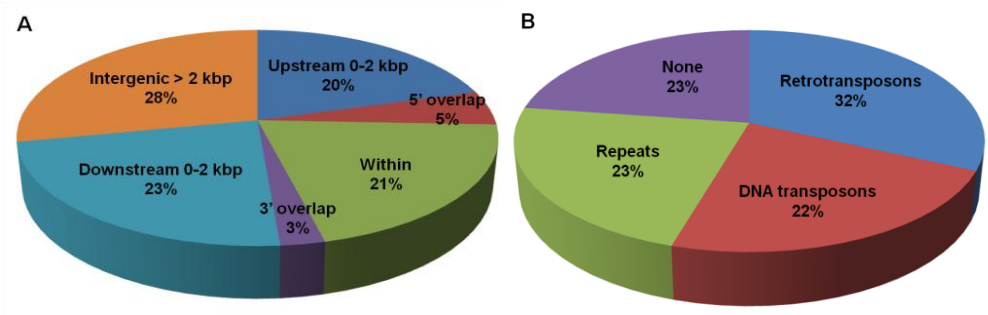


Figure 2: Classification of DMRs according to genomic features. (A) DMRs location in relation to their position to the nearest genes: Upstream (> 2 kbp or between 0 and 2 kbp of the gene transcription starting site (TSS), 5' overlapping (in case the DMR overlaps to gene transcription starting site), Within (if the DMR falls completely within the borders of a gene), 3' overlapping, (in the case DMR overlaps the 3' end of an annotated gene), or Downstream (0 to 2 kbp or > 2 kbp from gene end). **(B)** DMRs location in relation to Repeat Masks annotation: Retrotransposons, Transposons and Repeats [(CGG)n and (GGA)n rich areas].

The assessment of DMRs methylation profile revealed that all methylation variations following salt exposure consisted of demethylation. Furthermore, a detailed analysis by BS-Seq of two DMRs validated the loss

of methylation upon salt stress (Figure 3). The three methylation contexts (CG, CHG, CHH) were present in both DMRs. In DMR2, the methylation was present mainly in the CHH context, while in DMR15 there was a predominance of the CHG context. This analysis also revealed variation in methylated cytosines content among different DMRs. The DMR2 has considerably less methylation than DMR15 (2.012% and 5.510% respectively) but both DMRs suffered a loss of methylation upon salt stress (1.066% and 4.494% for DMR2 and DMR15, respectively).

3.4.3. Differentially Methylated Regions (DMRs) may have a role in gene regulation upon salt stress

Based on the hypothesis that DMRs may generate specific methylation landscapes with consequences at gene regulation we identified a set of genes located in the vicinity of the DMRs (Table 2) and performed expression studies for specific genes selected according to DMR position in relation to genes. For example, the DMR2 is located upstream two genes (Figure 4A) as well as the DMR9 (Figure 4C). Other three genes were selected for expression studies since they have a DMR located within the gene body (Figure 4B, D, E). Statistical data analysis by two-way ANOVA revealed a significant interaction between gene and treatment ($F(5,30)=55.767$; $p<0.001$) as well as between treatments ($F(1,30)=63.817$; $p<0.001$). The genes coding for a DNA binding protein and a retrotransposon (Figure 4A and D) presented the higher induction under salt stress. Interestingly, the gene expression induction by salt stress was always observed in genes having DMRs located in their promoters.

Furthermore, a gene ontology analysis was performed to identify predominant functions or similar roles between genes located nearby DMRs. Most of the proteins play two main functions, namely kinase activity and DNA and nucleotide binding (Figure S3A). Concerning the cellular component, the majority of the proteins are membrane linked and located on

plastids and thylakoids (Figure S3B). Finally, the two most prevalent biological processes are protein modification and signal transduction (Figure S3C). These results point for a role of DMRs in response to salt stress possibly by influencing the transcriptional activity of specific genes encoding several classes of proteins, preferentially those involved in signal transduction pathways and in photosynthesis.

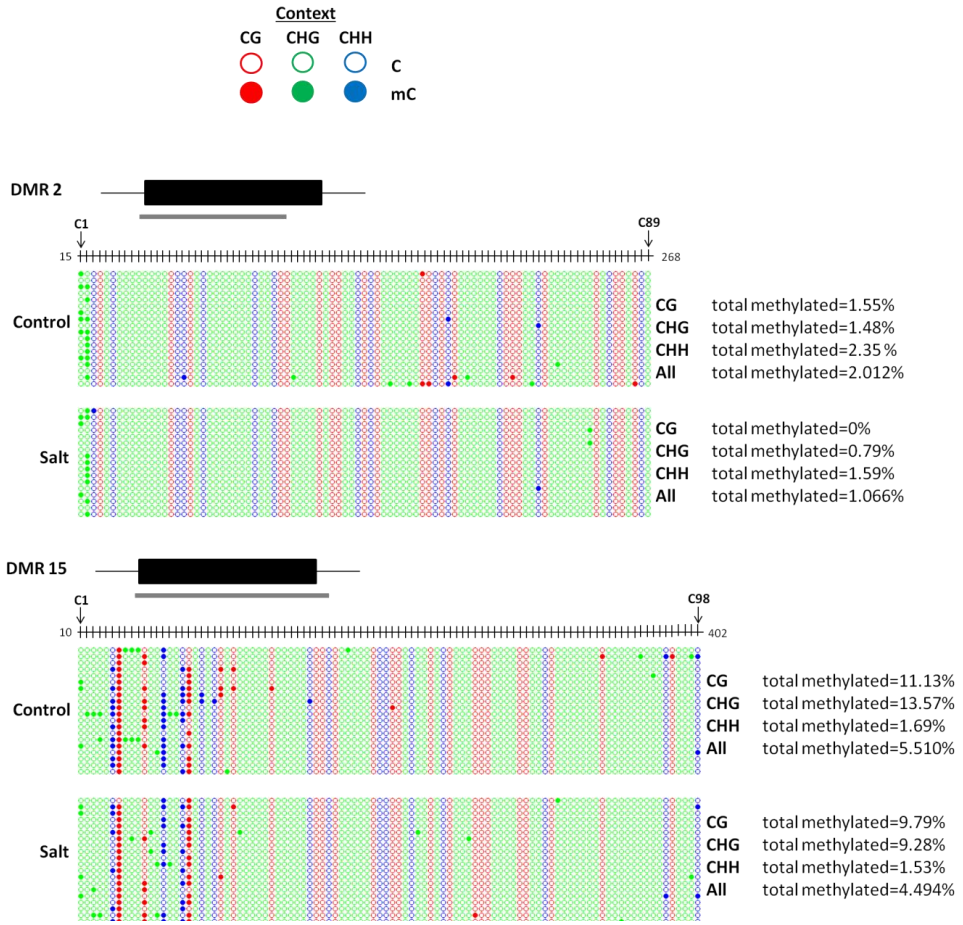


Figure 3: Bisulfite sequencing (BS) analysis for DMR2 and DMR15. The bisulfite analyzed region is indicated by a grey line under the DMR (black box). Positions of the first and last cytosines analyzed by bisulfite are indicated. Cytosine methylation contexts are symbolized by red circles for CG, green circles for CHG and blue circles for CHH (H=A, T or C). Total methylation (at the different contexts and globally) is also presented.

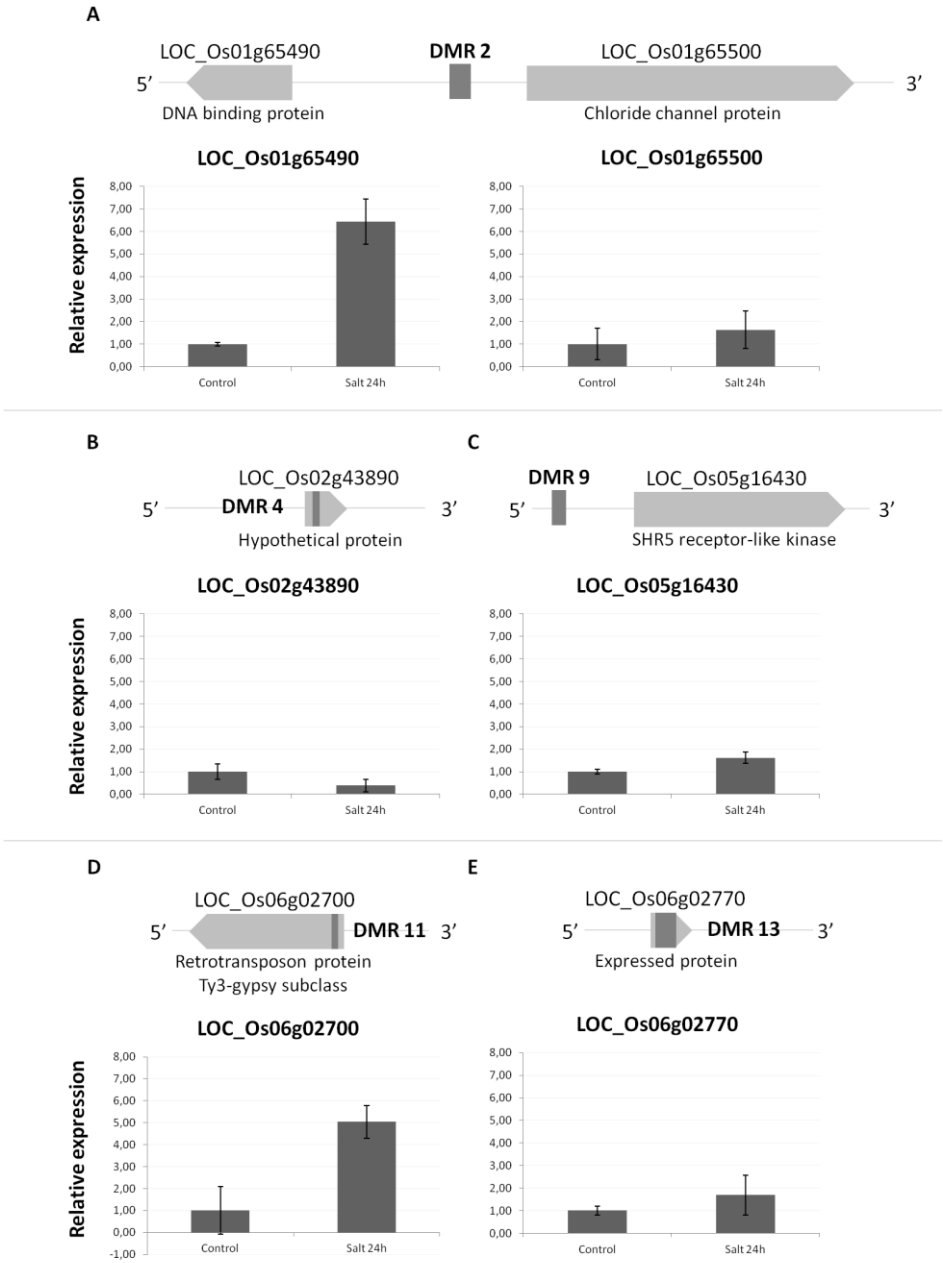


Figure 4: Expression studies of genes nearby DMRs by quantitative real-time qPCR. Genes containing DMRs at promoter regions are shown in **(A)** and **(C)** while genes containing DMRs within the coding region are shown in **(B)**, **(D)** and **(E)**. The mean expression value of control was normalized to 1 and the other mean values represent fold changes in expression of 3 technical replicates. The graphics show the result of one representative biological assay, from a total of 3 different replicates.

3.5. Discussion

In rice, the exposure to salt stress has been connected to chromatin remodelling as well as to changes in DNA methylation patterns (Santos *et al.*, 2011; Wang *et al.,* 2011: Karan *et al.*, 2012; Ferreira *et al.,* 2015). The methylome of leaves tissues from the salt-tolerant rice variety Pokkali was evaluated by high-throughput sequencing of immunoprecipitated anti-5-mC DNA (MeDIP-Seq), which revealed methylated areas mostly dispersed throughout the rice genome. Other studies in rice showed that centromeres are not densely methylated and possess euchromatic subdomains that allow gene transcription (Nagaki *et al.,* 2004; Wu *et al.,* 2011). Contrastingly, in Arabidopsis methylation has been described as particularly dense at centromeric regions (Haupt *et al.,* 2001; Zilberman *et al.,* 2007; Lister *et al.,* 2008). Our initial hypothesis was that certain genomic regions may be preferentially selected for differential methylation between control and salt stress samples. By adopting strict filtering criteria along MeDIP-seq processing data, 53 Differentially Methylated Regions (DMRs) were identified between control and salt stress. Interestingly, DMRs were located relatively far from centromeres and most of them (> 70%) overlap with repeat masks falling either within or in the vicinity of genes (less than 2kbp away). The preferential mapping of DMRs within or close to genes and far from centromeres was also observed in other plants such as maize (Candaele *et al.,* 2014) and poplar (Lafon-Placette *et al.,* 2013).

The functional meaning of DMRs is far from being understood. A plausible hypothesis is that DMRs may modulate chromatin landscape influencing the transcriptional competence of particular genomic regions. In humans, tissue specific DMRs correlated either negatively or positively with gene expression (Wan *et al.,* 2015). Here, we investigated the potential role of salt stress related DMRs in regulating the expression of stress responsive genes in rice. The methylation loss at DMRs upon salt stress detected in this study is in agreement with previous reports of demethylation caused by salt

stress in rice (Wang *et al.,* 2011; Karan *et al.,* 2012; Ferreira *et al.,* 2015). The finding of a correlation between hypomethylated DMRs and transcriptional activity of nearby genes suggests that DMRs may have a role in gene expression regulation. Additionally, it may well be possible that DMRs may also act as enhancer-like regions able to impact the expression of genes even when these are located far apart (Blackwood and Kadonaga, 1998).

The traditional correlation of DNA methylation with gene repression is being increasingly questioned. Studies have been mainly focused on the impact of DNA methylation in the promoter regions and in general DNA methylation in the promoter has been widely correlated with gene repression (Li *et al.,* 2008; Zhang *et al.,* 2008; He *et al.,* 2010; Li *et al.,* 2012). A still puzzling question concerns the functional meaning of methylation at the coding region level. In tomato, certain methylation contexts within coding region can have an important role in gene expression regulation (González *et al.,* 2011). In poplar, the methylation within the coding region has a more negative effect on transcription than the promoter-gene methylation (Vining *et al.,* 2012). Contrastingly, in rice, methylation within the coding region was shown to have a larger effect on transcription than the methylation at the promoter (Li *et al.,* 2008; Li *et al.,* 2012). Still, regarding methylation in the coding region, we found DMRs located either in the exons, introns or even in the exon/intron transition, which may eventually suggest a possible involvement of DNA methylation in splicing mechanisms. Although this link is still much unexplored in plants, recently it was reported the involvement of various splicing factors in different steps of the RdDM pathway (Huang and Zhu, 2014). Taken together, our results do not allow establishing a direct association between DMRs location (promoter or coding region) and gene activity since genes having DMRs on their promoter regions were activated by salt stress while genes with DMRs within the coding region were either activated or repressed.

A preliminary functional analysis of genes adjacent to DMRs was further achieved through a gene ontology examination. We found a set of overrepresented GO terms related to proteins with binding and kinase activity, membrane-linked, which match the most distinctive attributes that categorize genes involved in plant physiological and biochemical responses to salt stress, namely signal transduction, ion transport, biosynthetic, metabolic and protein modification processes (Hasegawa *et al.,* 2000). For example, microarray data revealed that the LOC_Os09g15480, encoding a serine/threonine-rich protein, is repressed by salt stress in 'Pokkali' roots (Hruz *et al.,* 2008). These plant-specific serine/threonine protein kinases can lead to Na^+ detoxification (Tester and Davenport, 2003) which is a mechanism occurring in salt stress responses. Therefore, this gene can be considered a putative target in salt tolerance mechanisms.

In the present study, salt stress-specific DMRs were identified and further characterization showed their impact on the transcriptional activity of nearby genes. We propose that DMRs can act as regulatory hotspots for gene transcription by placing chromatin structure in a more prepared state for efficient response to stress conditions.

3.6. Acknowledgments

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3.7. References

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Vining KJ, Pomraning KR, Wilhelm LJ, Priest HD, Pellegrini M, Mockler TC, Freitag M, Strauss SH (2012) Dynamic DNA cytosine methylation in the

Populus trichocarpa genome: tissue-level variation and relationship to gene expression. *BMC Genomics* **13:** 27

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3.8. Supporting information

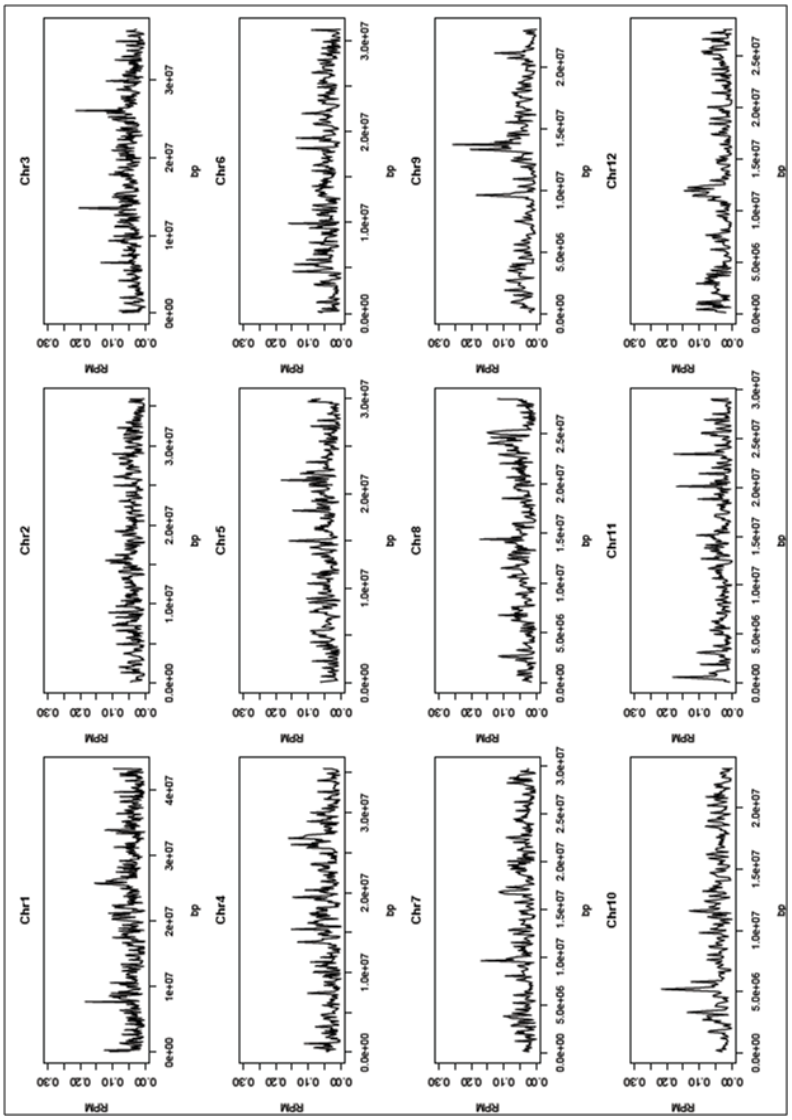
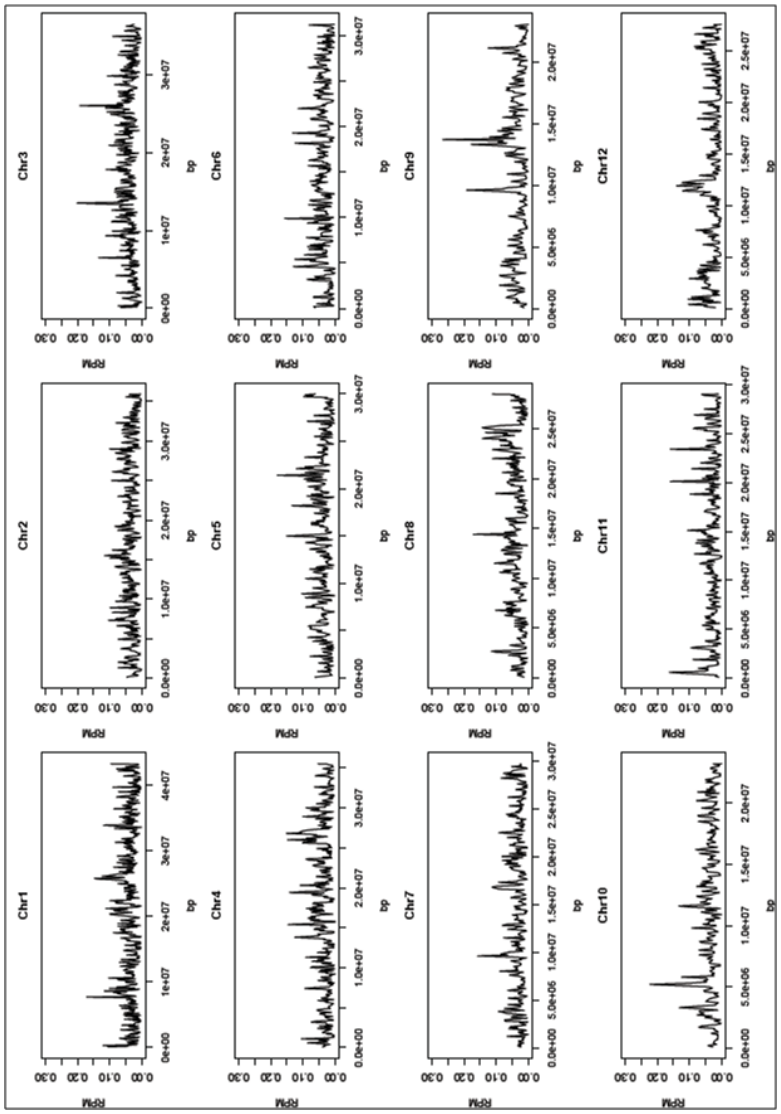


Figure S1: Chromosome-level view of DNA methylation in control (A) and salt stress (B) conditions. The chromosome read coverage plots were based on the average RPM over 100000 bp windows.


Chr1
Chr2
Chr3
Chr4
Chr5
Chr6
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Chr8
Chr9
Chr10
Chr11
Chr12
RPM
bp

B

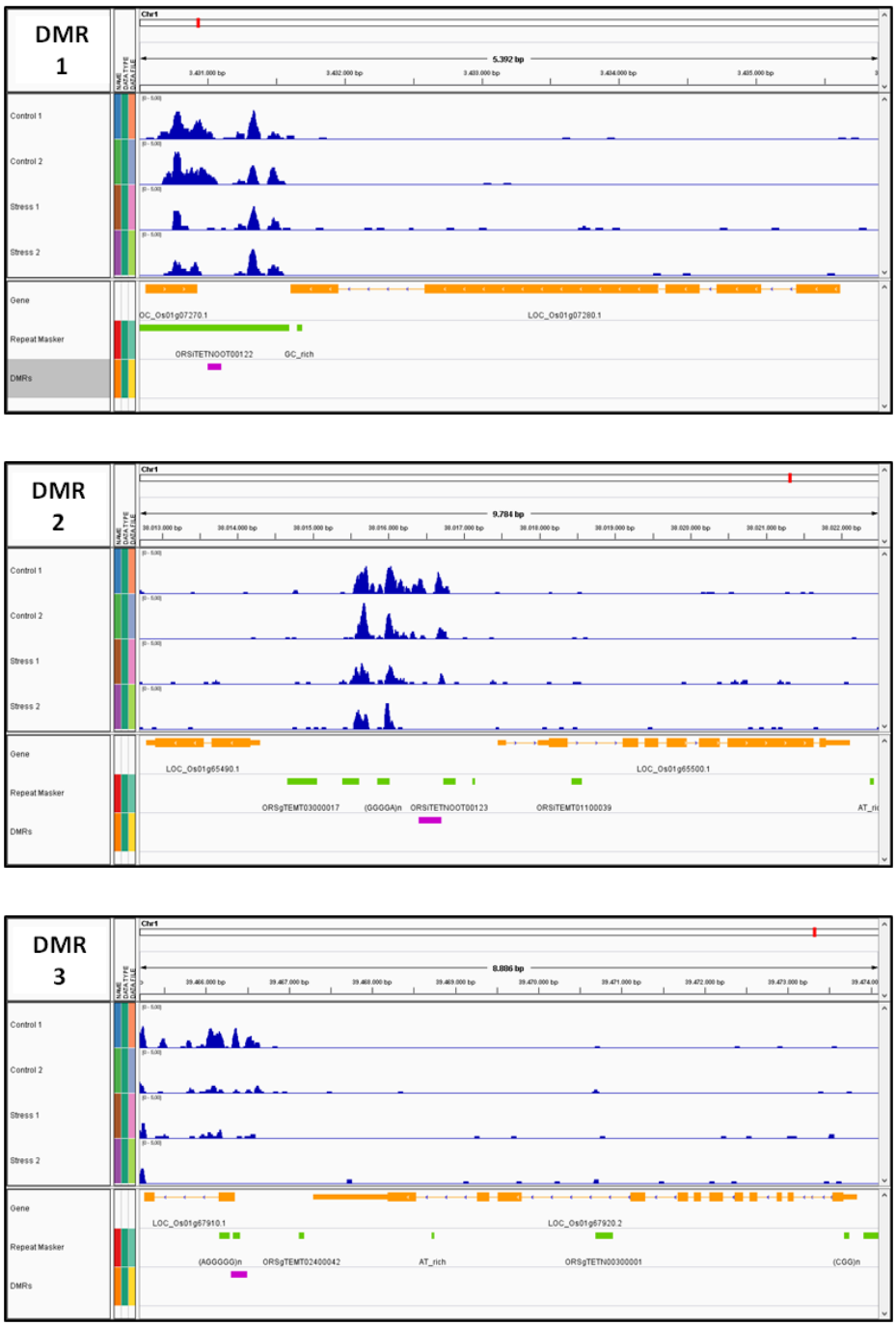
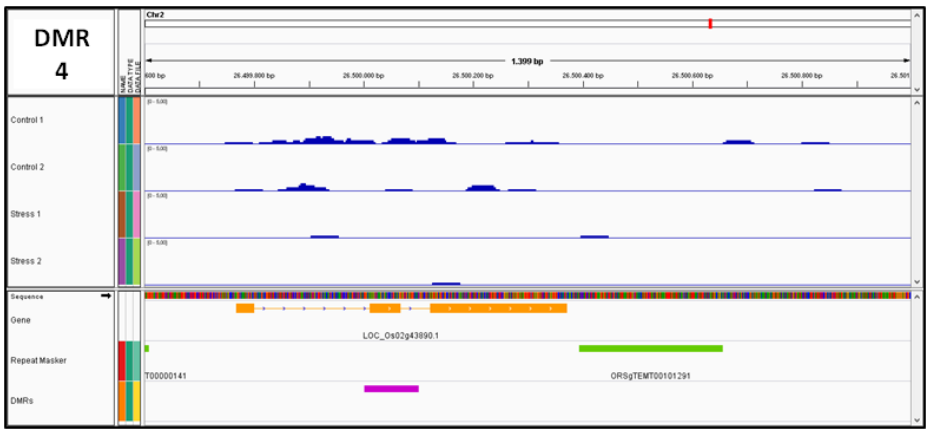


Figure S2: Methylation status of all DMRs identified between control and salt stress conditions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Chr2
1,399 bp
Control 1
Control 2
Stress 1
Stress 2
Sequence
Gene
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Repeat Masker
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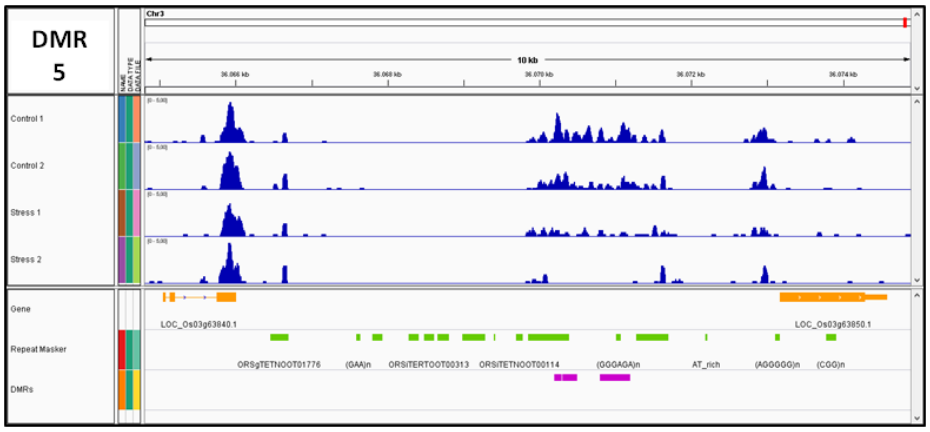
ORSgTEMT00101291
DMRs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Control 2
Stress 1
Stress 2
Gene
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LOC_Os03g63850.1
Repeat Masker
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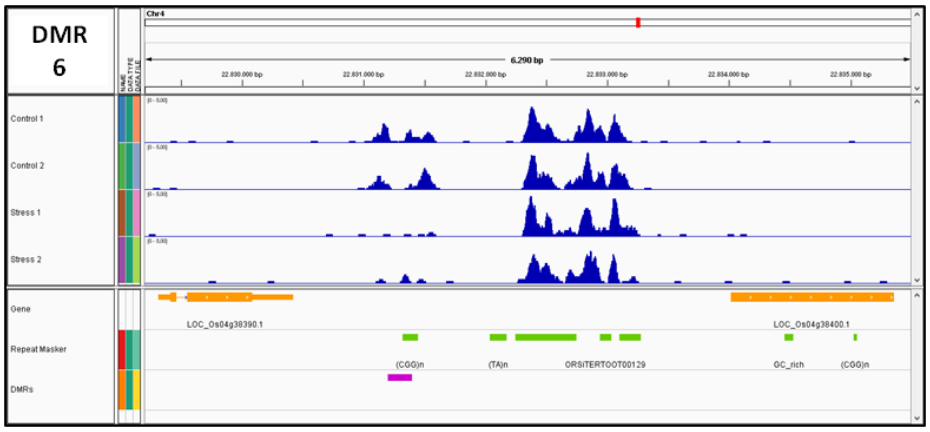
ORSiTERTOOT00313
ORSiTETNOOT00114
(GGGAGA)n
AT_rich
(AGGGGG)n
(CGG)n
DMRs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6
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Control 2
Stress 1
Stress 2
Gene
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Repeat Masker
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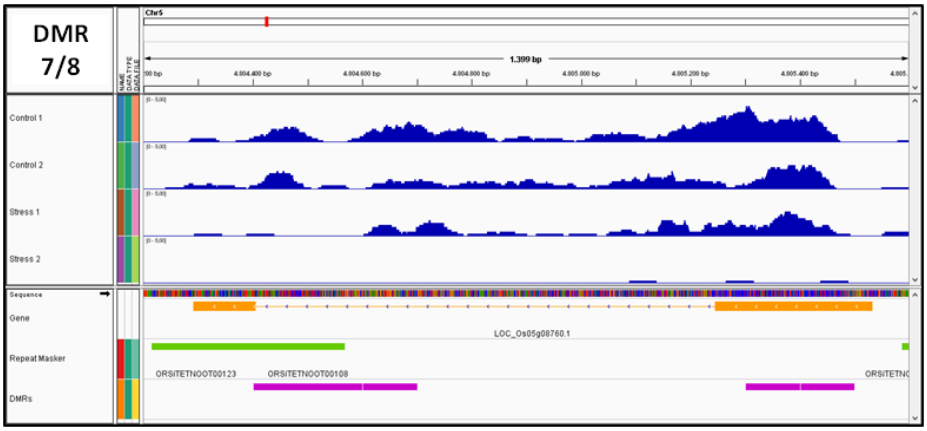
ORSiTERTOOT00129
GC_rich
(CGG)n
DMRs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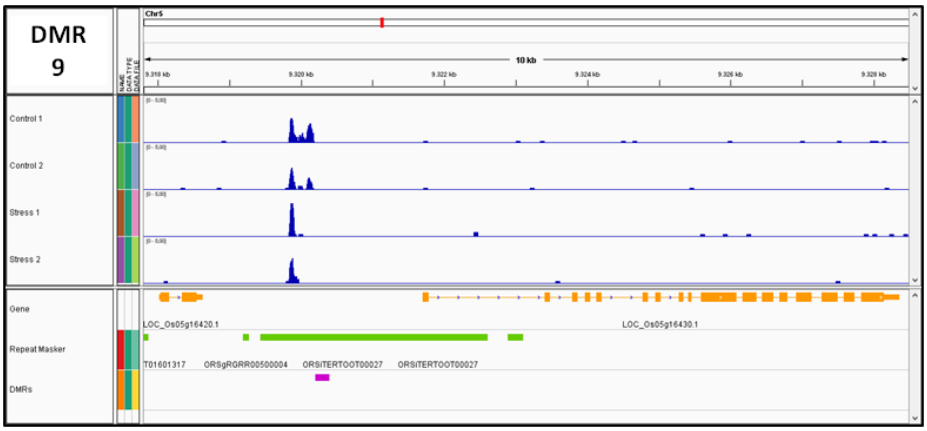
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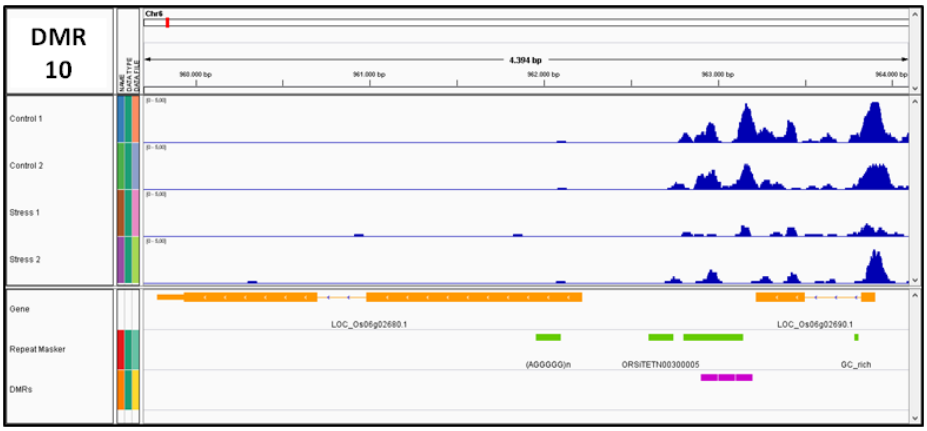
DMRs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Control 2
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Stress 2
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Repeat Masker
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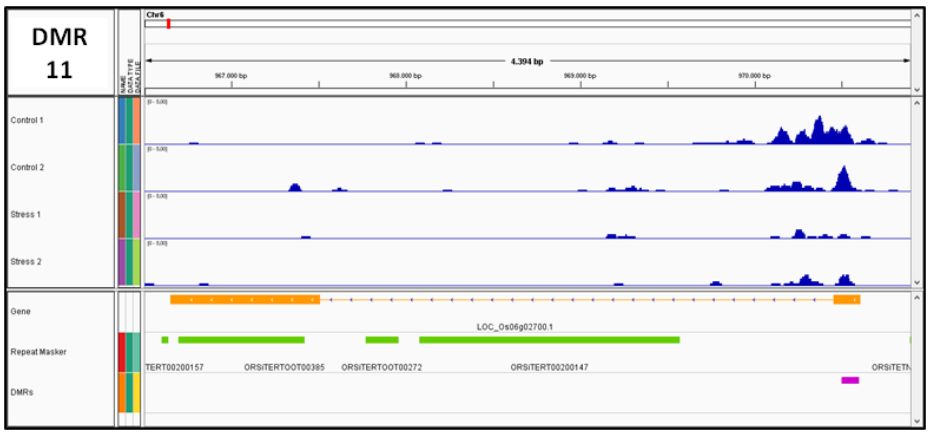
GC_rich
DMRs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968.000 bp
969.000 bp
970.000 bp
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Control 2
Stress 1
Stress 2
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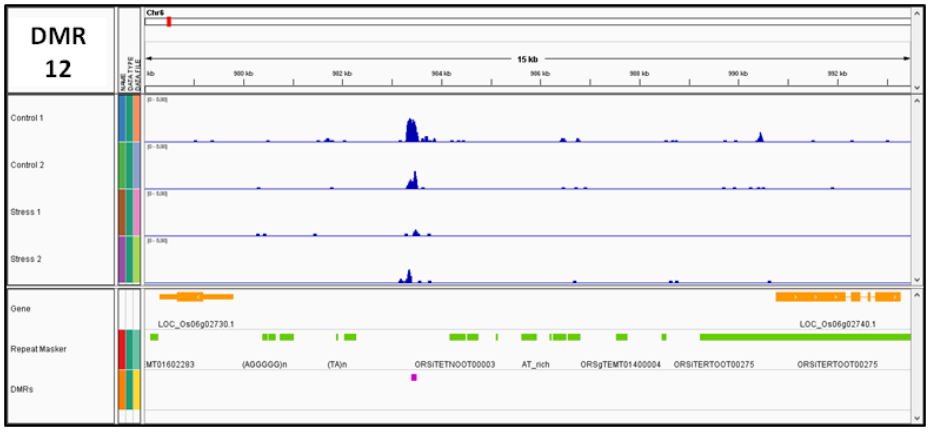
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912 kb
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Control 2
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Stress 2
Gene
Repeat Masker
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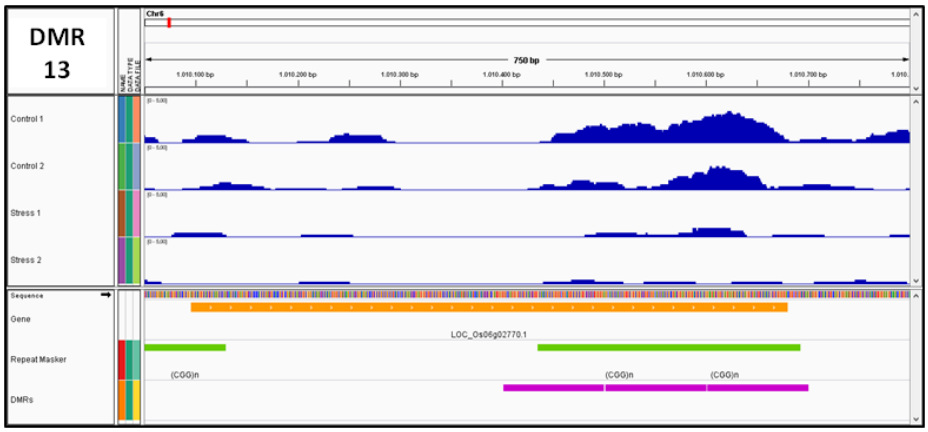
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13
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1,010,700 bp
Control 1
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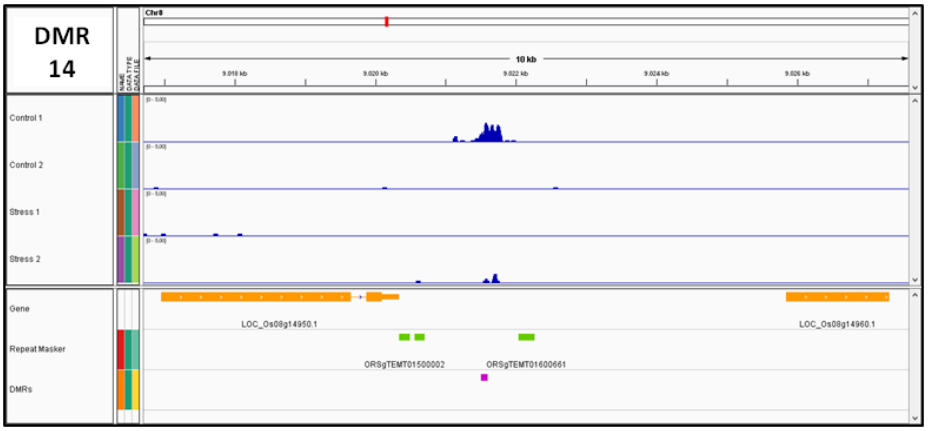
(CGG)n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Control 2
Stress 1
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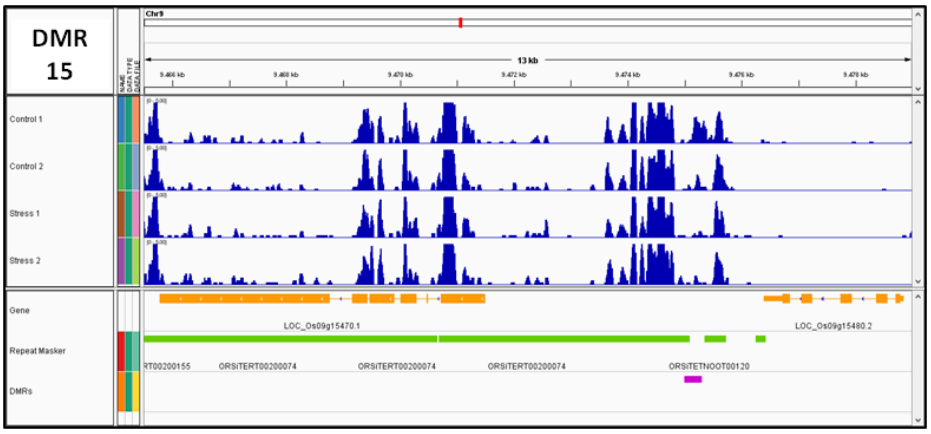
ORSgTEMT01600661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Control 1
Control 2
Stress 1
Stress 2
Gene
Repeat Masker
DMRs
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LOC_Os09g15480.2
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ORSITERT00200074
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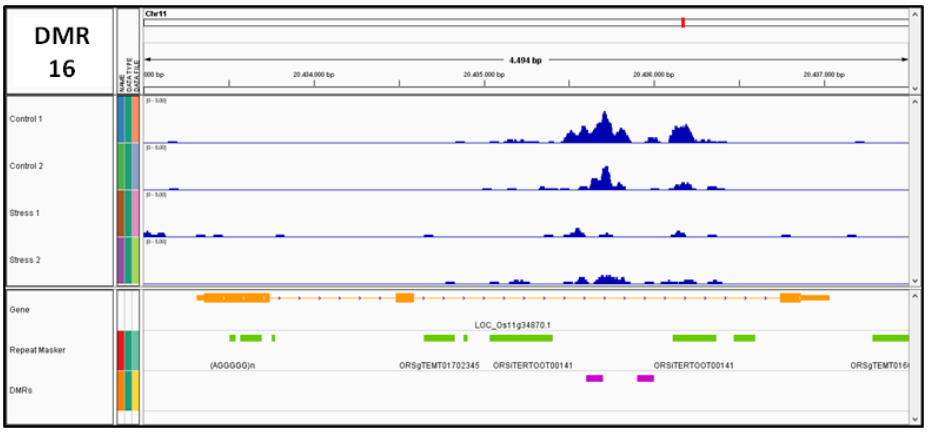
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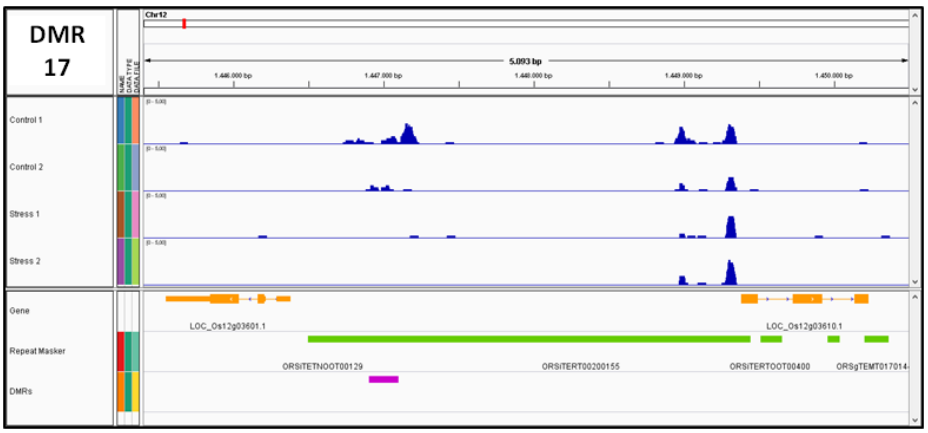
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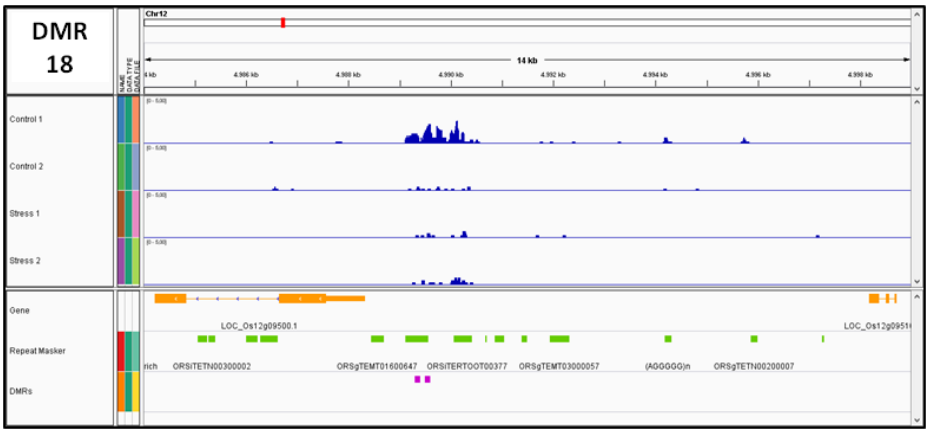
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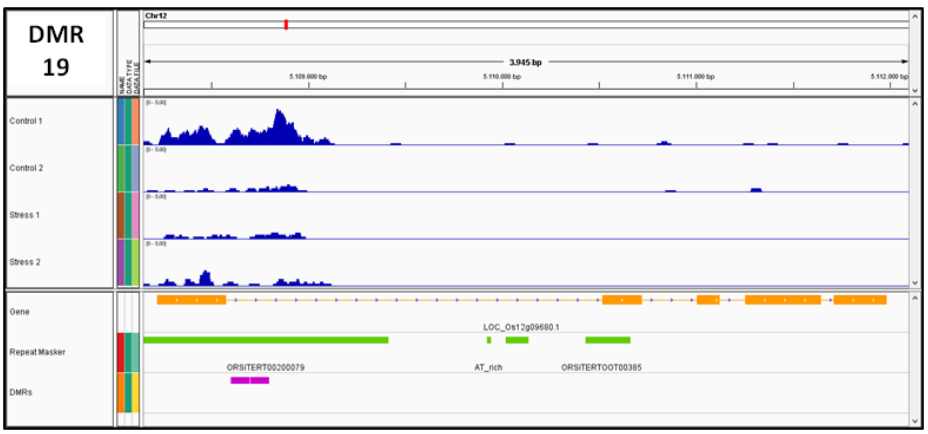
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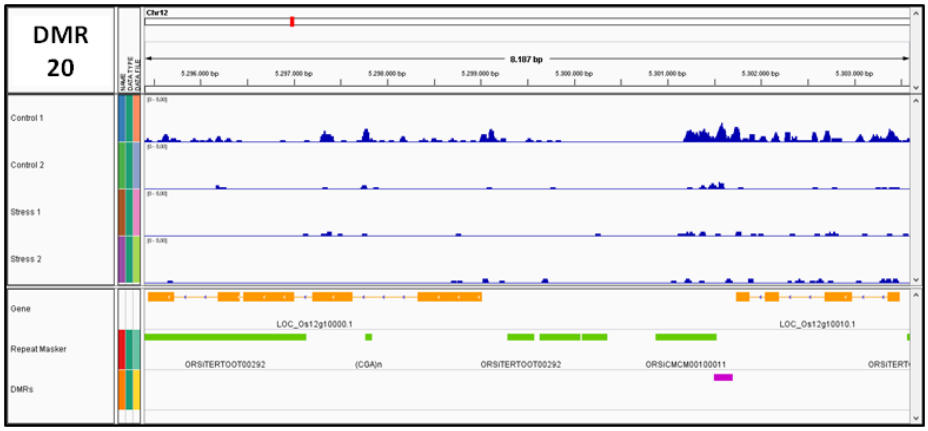
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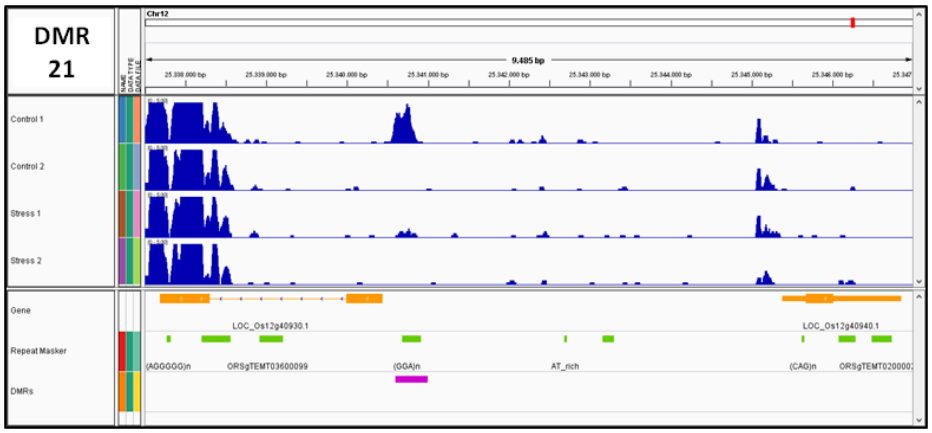
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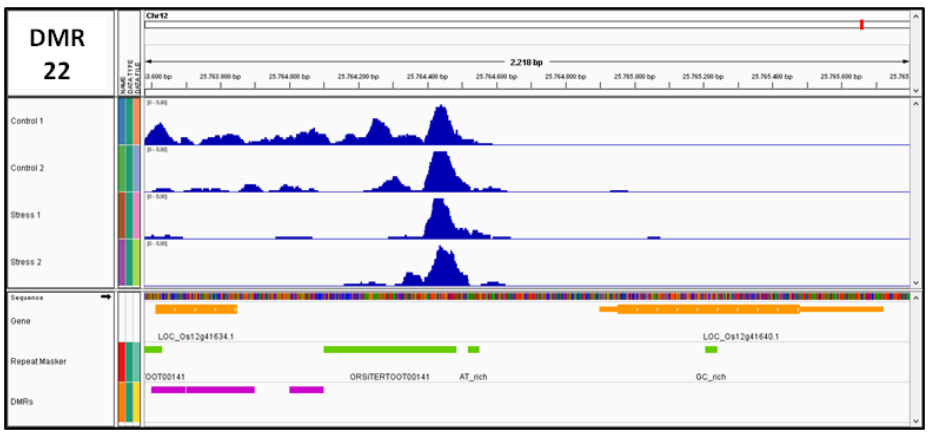
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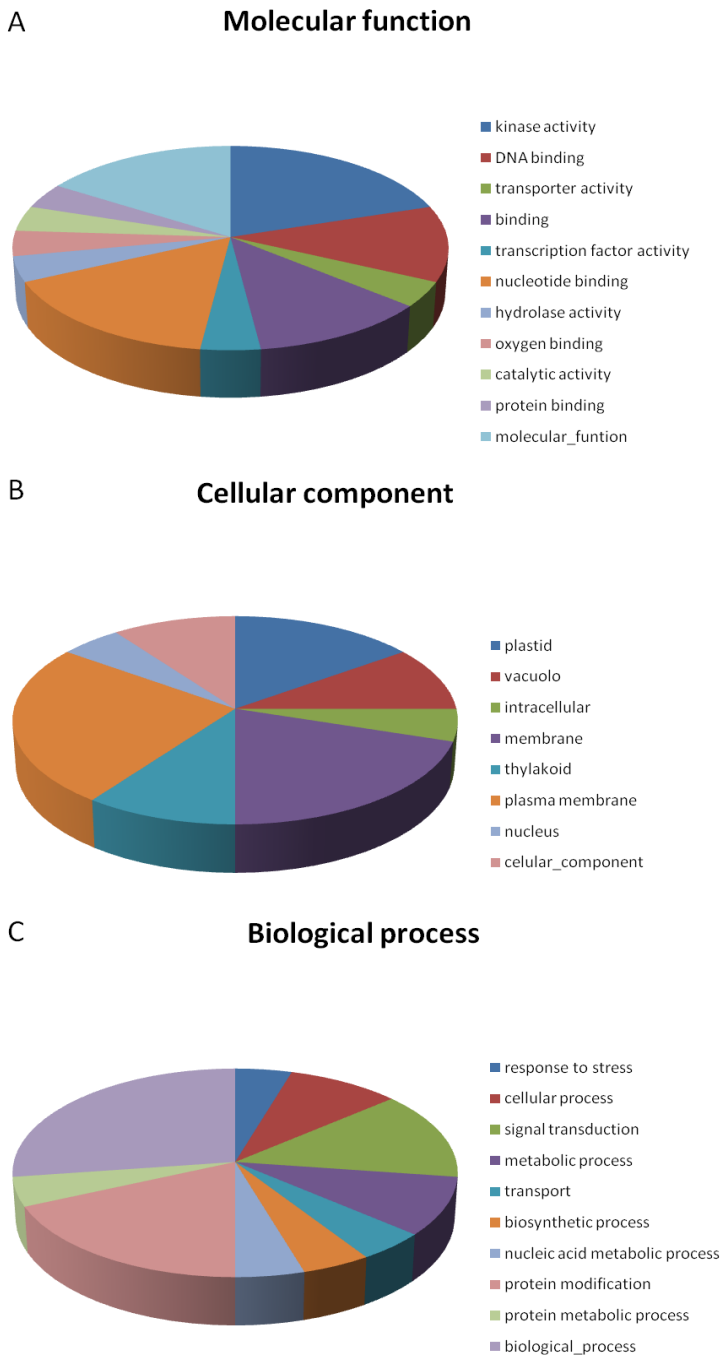

Figure S4: Gene Ontology (GO) analysis.

S1 Table: List of primers used for BS-PCR analysis.

DMR	Primer forward	Primer reverse
DMR 2	GTGGATGATGGGTGYYAAAGTAGGA	TCCTCTTCTCTCCTCTTCCATTA
DMR 15	TGTTATTTGYATTGATTTAATAGGG	ACTACCTCCCTCTCTCTCTCA

S2 Table: List of primers used for expression studies of genes located nearby DMRs

Genes	Primer forward	Primer reverse
LOC_Os01g65490	TTGTACATGCAGGGCACCTC	GTACGTCGACTGGTCCAGAG
LOC_Os01g65500	GCGCTTCTCTGGAGGACATT	CGCTGCGGCAGTACTCTATT
LOC_Os02g43890	TGTCACAGACCGCTACATCC	GAAGAATGCACCCACTCCGT
LOC_Os05g16430	AGAGCCTACAAACCTTGGGG	TTCCATTTGAGGCCAGGGAA
LOC_Os06g02700	TGATGAAGTTCTTCTGGCCG	ACAACAATATCGGCTCGGGG
LOC_Os06g02770	GATTTGGGTGTGGCCGAGTG	CACCAAGCAGCGAGCAAC

Chapter 4

Enrichment of euchromatin marks and nucleosome repositioning underlies *OsRMC* activation by salt stress

Liliana J. Ferreira contributed to the planning and execution of all experiments presented in this Chapter, as well as to the discussion, interpretation and preparation of the manuscript.

Part of this work has been accepted for publication in the book under the working title "Rice", ISBN 978-953-51-4979-8, and is being written for submission.

Ferreira LJ, Ravasco S, Figueiredo D, *et al.* (2016) Deciphering histone modifications in rice by chromatin immunoprecipitation (ChIP) and *in situ* immunofluorescence

Chapter 4:
Enrichment of euchromatin marks and nucleosome repositioning underlies *OsRMC* activation by salt stress

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4.1. Abstract

Soil salinity is a major environmental constraint for crops, with an obvious negative impact on production levels, sustainability and world economy. Plants possess intricate mechanisms to assure an effective control of stress-responsive genes, with gene transcription being regulated at multilayered levels involving chromatin structure, histone modifications and transcription factors (TFs). This work focused on the role of specific marks of histone modifications in the regulation of a gene highly induced by salt stress, *OsRMC*. The expression level of *OsRMC* was analysed under modified epigenetic control, either using the hypomethylating agent 5-azacytidine (5-AC) in seed germination, or by affecting epigenetic regulators with T-DNA-mediated mutagenesis. The increased expression of *OsRMC*, in the epimutants and after 5-AC induced hypomethylation, showed that this gene is under strong epigenetic regulation. Moreover, Chromatin immunoprecipitation assays with specific histone modifications marks revealed a differential enrichment of euchromatic marks depending on the promoter region. The chromatin domain in which the transcription factors were found to bind was particularly enriched with histone modification euchromatin marks, suggesting that nucleosome repositioning may play a role in *OsRMC* gene activation by salt stress.

4.2. Introduction

The gene expression regulation in eukaryotes depends largely on the status of chromatin structure. Chromatin folding can interfere with the recognition of specific promoter sequences by transcription factors and RNA polymerases (Santos *et al.,* 2011a). The chromatin accessibility to the transcriptional machinery is mainly regulated by proteins known as chromatin-modifying coactivators including chromatin remodeling complexes that use the ATP hydrolysis-derived energy to destabilize chromatin, leading to histone displacement or even removal (Papamichos-Chonakis and Peterson, 2013). In addition, chromatin accessibility is greatly influenced by post-translational histone modifications such as acetylation, methylation, phosphorylation or ubiquitinylation, which can alter the physical properties of the chromatin or regulate the binding of non-histone proteins (Workman and Kingston, 1998; Kornberg and Lorch, 1999; Rothbart and Strahl, 2014). The 'histone code' postulates that specific combinations of histone variants and post-translational modifications (PTMs) play a key role in the modulation of active and inactive chromatin states via covalent modifications of histone tails (Strahl and Allis, 2000; Jenuwein and Allis, 2001; Berger, 2007; Kim *et al.,* 2010). Post-translational histone modifications such as histone acetylation or deacetylation are modulated by histone acetyltransferases (HATs) and histone deacetylases (HDACs), respectively. The HATs and HDACs exist as multiprotein complexes that are linked to gene transcription mechanisms functioning as transcription co-activators or co-repressors and have relatively low specificity, since each enzyme can modify different residues (Yang and Seto, 2007). Contrastingly, the histone methyltransferases (HMTs) and histone demethylases (HDMs), that mediate histone methylation and demethylation, are highly specific since they may restrict to the modification of one single lysine in a single histone (Bannister and Kouzarides, 2005; Kouzarides, 2007). The rice genome encodes at least eight HATs, fourteen HDACs, twelve HMTs and twenty HDMs (Fu *et*

al., 2007; Qin *et al.,* 2010; Liu *et al.,* 2012; Hou *et al.,* 2015). The OsHATs are constitutively expressed in rice, and their expression can be regulated by hormones and abiotic stresses, including salt stress (Liu *et al.,* 2012). Some HDACs have been reported as salt stress responsive and others have been related to plant development (Hu *et al.,* 2009). Gene expression regulation is influenced by the histone modification landscape, namely by the spatial distribution of specific modifications along the gene promoter region (Bernstein *et al.,* 2007; Berger *et al.,* 2007; Kouzarides 2007; Li *et al.,* 2011). In Arabidopsis, abscisic acid (ABA) treatments and imposed salt stress heve led to an enrichment in the H3K9K14ac and H3K4me3 activators marks, and to a decrease of the H3K9 dimethylation repressive mark at specific genes responsive to abiotic stress (Chen *et al.,* 2010). Also in Arabidopsis, the *HKT1* gene that is transcriptionally induced by salt stress, suffered a decrease of the H3K27me3 repressive mark (Sani *et al.,* 2013). In rice, an increase of acetylation of histone H3 was positively correlated with the increased expression of the genes *ADH1* and *PDC1* after exposition to submergence stress (Tsuji *et al.,* 2006). Also in rice, the activation of some drought stress-responsive genes was correlated with changes in the H3K4me3 mark (Zong *et al.,* 2013). All together, these reports have shown that fluctuations of specific histone modification marks are linked to transcriptional regulation of specific gene networks underlying specific stress types.

In this work, we addressed specific histone modifications, namely lysine acetylation (H3K9ac and H4K5ac) and trimethylation (H4K20me3), along specific promoter regions of *OsRMC* gene, which is highly induced by salt stress. The *OsRMC* expression level was increased when epigenetic levels were deliberately altered, particularly in the case of induced global hypomethylation by seed germination in 5-AC and also in the case of knock-out mutations for a histone acetyltransferase or DNA methyltransferase. The gene activation was correlated with fluctuations at the histone modifications

landscape along specific gene promoter regions. We detected a global enrichment of the euchromatic marks H4K5ac and H4K20me3 after salt stress particularly at the promoter region where transcription factors can bind. Deciphering the landscape of chromatin accessibility in response to stress may bring new insights to the comprehension of epigenetic regulation of gene networks underlying salt responses.

4.3. Materials and methods

4.3.1. Plant material, growth conditions and stress treatments

Wild type rice (*Oryza sativa L.* ssp *japonica* AA, 2n=24) cv. Nipponbare and cv. Dongjin, as well as Dongjin plants with mutations for epigenetic regulators, were grown at the ITQB green houses and used in these experiments. The rice mutants selected are T-DNA insertion lines (4A-01884, 3A-08043), originally developed by Gynheung An (Kyung Hee University, Crop Biotech Institute, Korea) carrying mutations for a histone acetyltransferase (*oshac704*) and a DNA methyltransferase (*osdrm2*). The genotyping was performed as described in Ferreira *et al.* (2015).

Rice seeds were surface disinfected in a Benlate solution (0.1%) for 30 min at 50ºC, rinsed with sterile water, soaked in 70% ethanol for 1 min and washed with a solution of 2% sodium hypochlorite containing 0.02% Tween 20 for 30 min. After several washes with sterile water, seeds were germinated on Petri dishes containing 3MM paper embedded in sterile water, in the darkness, for 3 days, at 28ºC. Germinated seedlings were transferred to glass tubes containing Yoshida's medium (Yoshida *et al.,* 1976) and allowed to grow in a growth-chamber under 28ºC/24ºC in a 12h photoperiod regime 500 $\mu Em^{-2}s^{-}1$ and with 70% humidity.

The salt stress was imposed on 14-days-old rice seedlings by supplementing Yoshida's medium with 150 mM NaCl during 5h and 24h. For applying the 5-AC treatment, the Yoshida's medium was supplemented with

80 µM of 5-azacytidine (Sigma) and the solution was made fresh and changed daily as referred in Santos *et al.* (2002). Two experimental conditions were tested as shown in Figure 1A. The drug was imposed to seventh-days-old seedlings for 7 days and in the other, the drug was applied to eleventh-days-old seedlings for 3 days.

4.3.2. Imaging of H3K4me2 in interphase nuclei of root sections

Root-tips of 3-days-old seedlings grown in control conditions or under a saline solution of 150 mM NaCl were excised and fixed in 4% (w/v) formaldehyde freshly prepared from paraformaldehyde in PEM buffer (50 mM PIPES; 5 mM EGTA; 5 mM MgSO4; KOH pH 6.9) for 1h at room temperature and then washed in TBS for 10 min. Root tips were sectioned using a Vibratome Series 1000 (TAAB Laboratories Equipment Ltd, Aldermarston, UK) and allowed to dry on multi-well slides (Menzel-Glaser). Root sections of approximately 15–20 µm thickness containing about two cell layers showed good tissue preservation and integrity. The slides were pre-treated by washing in 3% Decon90 (detergent) for at least 1h, thoroughly rinsed with distilled water and coated with a freshly prepared solution of 2% (v/v) 3-aminopropyltriethoxysilane (APTES, Sigma) in acetone for 10 s and activated with 2.5% (v/v) glutaraldehyde (Sigma) in PBS for 30 min, rinsed in distilled water and air-dried. Prior to immunofluorescence, tissue sections were dehydrated in an ethanol series and digested with an enzyme mixture of cellulase 1.5% (w/v) (Onozuka R-10, Japan) and pectolyase 0.5% (w/v) (Sigma) in EB (0.4 mM citric acid; 0.6 mM trisodium citrate, pH 4.8) for 1h at room temperature. After washing 10 min with PBS, the sections were permeabilized with 0.1% Triton X-100 (v/v) in PBS for 3 min, washed again 10 min in PBS and dehydrated in an ethanol series and air-dried.

For *in situ* detection of H3K4me2, a rabbit monoclonal antibody against di-methylation of lysine 4 of histone H3 (1:500, Millipore) was used, followed by a secondary antibody (anti-rabitt Cy3 conjugated 1:500, Millipore). After

blocking with 1% (w/v) Bovine Serum Albumine (Roche) dissolved in PBS/0.1% Triton X-100 for 1h, the tissue sections were incubated with the primary antibody during 2h at 37ºC. After washing in PBS/0.1% Triton X-100 for 10 min, incubation with the secondary antibody was performed at 37ºC for 1h (or alternatively overnight at 4ºC). The nuclei were counterstained with DAPI (6 g/ml 4,6-diamidino-2-phenylindole, Sigma) for 10 min, rinsed briefly in PBS and mounted in Vectashield antifade solution (Vector Laboratories). Confocal optical section stacks (z-step size of 1 µm) were collected with a Leica TCS SP confocal microscope (Leica Microsystems, Heidelberg GmbH). The microscopy data were transferred to Image J and processed using constant parameters to minimize operator errors. The fluorescence intensity was measured in Z-projections obtained by using the maximum intensity of each stack individually. At least 30 nuclei were measured for each condition. The arbitrary units correspond to the values of the Raw Integrated Density measurement. Finally, Adobe Photoshop 5.0 (Adobe systems Inc., Mountain View, CA) was used for image composition.

4.3.3. Protein extraction and immunoblotting analysis

Root-tips of 3-days-old seedlings grown in control conditions or under a saline solution of 150 mM NaCl were excised and homogenized in Laemmli sample buffer (#161-0737 Bio-Rad). Protein concentration was determined through the Bradford assay (Bradford, 1976). Protein samples were resolved on 16% acrylamide-SDS gel electrophoresis and transferred to a nitrocellulose membrane by western blotting. The blots were probed with the rabbit monoclonal antibody anti-H3K4me2 (#07-030, Millipore) which is specific for dimethylated Histone H3 at lysine 4, diluted in TBS according to the manufacturer's instructions. Proteins were visualized using a secondary antibody goat anti-rabbit ECL conjugated, diluted 1 in 5000 in 5% BSA/TBS-T.

4.3.4. Gene expression analysis

Gene expression studies were performed on wild type rice plants of cv. Nipponbare and cv. Dongjin, and on Dongjin mutants obtained from mutagenesis with T-DNA. The T-DNA inserts affected epigenetic regulators namely a histone acetyltransferase (oshac704) and a DNA methyltransferase (osdrm2). Total RNA was extracted from leaf tissues of twelve rice seedlings grown for 14 days exposed to salt stress or to 5-AC drug. The RNA extraction procedure followed the manufacturer's instructions (Zymo Resarch). The isolated total RNA was treated with TURBO DNA-free (Ambion) to eliminate any possible DNA trace. The synthesis of cDNA from the RNA template was made from 4 µg of total RNA using the Randon Hexamer primer and according to the kit manual instructions from the Transcriptor High Fidelity cDNA Synthesis Kit (Roche). Semi-quantitative RT-PCR was used to analyze the *OsRMC* (LOC_Os04g56430) transcript activity in rice cv. Nipponbare, in control, salt stress or 5-AC conditions. The PCR running conditions included one cycle at 95ºC for 3 min and 40 cycles of amplification at 95ºC for 20 s, 56ºC for 20 s and 72ºC for 30 s. Real-time quantitative qPCR using the LightCycler 480 system (Roche) and the SYBR Green I Master mix (Roche) was performed for transcription analysis of the *OsRMC* gene in the mutants. The PCR running conditions were as follows: one cycle at 95°C for 5 min and 45 cycles of amplification at 95°C for 10 s, 52°C for 10 s and 72°C for 10 s. All qPCR experiments were performed on at least two biological replicates and the CT values were calculated from means of three technical replicates. The relative quantification of *OsRMC* gene was calculated with kinetic PCR efficiency correction using the comparative Ct method (2(-ΔΔCt) to determine the relative expression of transcripts relative to an endogenous control. The rice ribosomal gene 25S rRNA was used as endogenous gene to normalize the relative expression of the target transcripts. The primers used are listed on Table S1.

4.3.5. Chromatin immunoprecipitation (ChIP) assay

The ChIP assays were performed with 14 days-old rice leaves (cv. Nipponbare). Two days before applying salt stress, the plants were transferred from glass tubes to larger containers in order to facilitate the manipulation and minimizing the harvesting time of the biological material. The ChIP procedure was based on that described by Haring *et al.* (2007) but including optimizations to adapt it to specificities of the rice leaf tissues. The whole process of ChIP is described in detail below and the list of solutions used is indicated on Table S2.

Chromatin crosslinking optimization

The crosslink process is illustred in Figure S1. Rice leaves of about 70 plants were submitted to vacuum infiltration. Rice leaves were quickly cut into small pieces of approximately 1 cm length and immediately mixed with 200 ml of the formaldehyde solution (Buffer A). The vacuum infiltration was applied for 2 min, during which vacuum was interrupted each time pressure reached 50 mbar, repeating this cycle for approximately 6-7 times. After stirring an additional 1 min without vacuum, the crosslink reaction was stopped by adding 20 ml of 2M Glycin. Vacuum was applied again for 5 min with pressure release every 30 s. The leaves were then thoroughly washed with water using a sieve and carefully dried with paper towels and at the end were frozen in liquid nitrogen until further use. The optimization of crosslink efficiency included testing two different concentrations of formaldehyde (0.8% and 1%) and performing the FAIRE (Formaldehyde-Assisted Isolation of Regulatory Elements) assay. Both concentrations assured a strong chromatin crosslink but the chromatin recovery after de-crosslink was approximately four times higher when using 0.8% p-formaldehyde and thus, it was established as the optimal concentration for chromatin crosslink (Figure S2). The qPCR, with any primer combination, was used to determine

the amount of DNA present in the samples. One hundred milligram of the infiltrated grinded material was resuspended in 1 ml of Extraction Buffer and vortexed for 1 min followed by centrifugation at 13000 rpm for 5 min at RT. Seven hundred microliters were transferred to a new tube and from these, 40 µl were decrosslinked by adding 620 µl of Buffer G and incubated overhight at 65ºC. One volume of Phenol:Chloroform (1:1, v/v) was added to the decrosslinked and to the remaining 660 µl samples. Both samples were then centrifuged at 16100 g for 5 min at 4ºC. Six hundred microliters from the supernatant were taken and chloroform (1 volume) was added. The samples were mixed and centrifuged as before. Four hundred and fifty microliters were taken from the supernatant and washed with 2 volumes absolute ethanol. The samples were centrifuged at 16100 g for 20 min at 2ºC. The supernatant was discarded and the pellet washed in 200 µl 70% ethanol, followed by centrifugation at 16100 g for 10 min at 2ºC. After drying, the pellet was ressuspended in 30 µl of water. For qPCR, 2-5 µl were used as a template, in triplicate.

Nuclei isolation and optimization of chromatin shearing

Ground material was resuspended in 40 ml of Buffer B in 50 ml tubes and incubated for 15 min at 4ºC with shaking. The suspension was filtered through 4 layers of miracloth into a new 50 ml tube and centrifuged at 2880 g for 20 min at 4ºC. The supernatant was discarded and the pellet was resuspended in 1 ml of Buffer C with a paint brush. The solution was transferred to a 1.5 ml tube and centrifuged at 12000 g for 10 min at 4ºC. The new pellet was resuspended in 300 µl of Buffer D and overlaid in 1.5 ml of Buffer D placed in a 2 ml tube. The samples were centrifuged at 16000 g for 1 h at 4ºC. The chromatin pellet was resuspended in 300 µl of Buffer E. A chromatin aliquot (20 µl) was taken for agarose gel analysis in order to compare shearing efficiency. The ideal size of chromatin fragments for an efficient immunoprecipitation process and subsequent qPCR amplification

should be around 300 bp (Haring et al., 2007). Here, chromatin sonication was performed in a Bioruptor sonicator (Diagenode) at 4ºC for 13 cycles of 30 s each and low intensity. The sheared chromatin was centrifuged at 16000 g for 5 min at 4ºC to pellet cell debris. The supernatant was transferred to a new 1.5 ml tube and a chromatin aliquot (20 µl) was taken to evaluate the shearing efficiency in agarose gel. Unsheared and sheared chromatin samples were decrosslinked overnight at 65ºC in 100 ml of Buffer G. The DNA was purified (High Pure PCR Product Purification Kit, Roche) and analysed on agarose gel to evaluate the efficiency of DNA shearing.

Chromatin Immunoprecipitation with antibodies for histone modifications

Prior to immunoprecipitation, the chromatin solution was pre-cleared. Two hundred microliters of chromatin were mixed with 1.8 ml of Buffer F and 30 µl of Protein A-Agarose (Santa Cruz, Biotechnology). The solution was incubated for 3-4 h at 4ºC under constant agitation, centrifuged at 500 g for 5 min at 4ºC and then the supernatant was collected. The immunoprecipitation was performed by incubating 400 µl of the pre-cleared chromatin with 5 µl of the selected antibody and 30 µl of Protein A-Agarose. The following commercial antibodies for histone modifications were used H3K9ac, H4K5ac and H4K20me3 (#07–352, #07-327 and #07–463, respectively; Millipore). Additionally, a no-antibody (NoAb) sample was used as a negative control. Another 40 µl of the pre-cleared chromatin was used as the input sample in which no Protein A-Agarose was added. The samples were incubated overnight at 4ºC with shaking and then centrifuged at 500 g for 5 min. After resting 5 min on ice, the supernatant was collected and frozen and the beads containing the immunoprecipitated chromatin were washed with 900 µl of the Low Salt buffer for 10 min under constant agitation at 4ºC and then centrifuged at 500 g for 5 min at 4ºC. After draining the supernatant, the immunoprecipitated chromatin was washed again with the High Salt buffer,

then with the LiCl buffer and finally twice with the TE buffer. The chromatin was allowed to air-dry to completely remove any TE buffer trace.

Reverse crosslink, DNA purification and RT-qPCR analysis

The bead-bound complexes were released by adding 200 µl of Buffer G to the immunoprecipitated chromatin and by 5 min vortexing followed by a brief centrifugation and incubation overnight at 65ºC. After brief centrifugation to pellet the agarose, the supernatant was collected and purified with the High Pure PCR Product Purification Kit (Roche). In the final step of the purification, the DNA was eluted in 80 µl and stored at -20ºC until further use. This step was also applied to the previously frozen input sample.

Precipitated genomic DNA was subjected to real-time qPCR with primer sets A-C encompassing the promoter region (A-C) of the *OsRMC* gene (Table S3). Five microliters of the eluted DNA were directly used as template in qPCR, in the following conditions: one cycle at 95°C for 5 min and 45 cycles of amplification at 95°C for 10 s, 52°C for 10 s and 72°C for 10 s. All qPCR experiments were performed on at least three biological replicates and the CT values were calculated from means of three technical replicates. An optimized qPCR setup developed by Haring *et al.* (2007) was followed. The optimization of qPCR primer sets was achieved by performing qPCR on serial dilutions of template DNA isolated from crosslinked and sonicated chromatin. The efficiency of amplifications should be as close to two as possible (meaning that a 2x dilution of the template should result in 1 Ct increase) and should remain constant over a wide range of template concentrations. The melting curve analysis enables to determine if the correct fragment is amplified. This extension of the PCR program consists on gradually increasing temperature with a continuous measuring of SYBR Green fluorescence. Since the PCR products will denature at a temperature specific for their size and sequence, this will be measured as a loss of fluorescence. Multiple PCR fragments in one reaction, or the presence of

primer-artefacts, will result in a step-wise decrease of fluorescence. Therefore, optimal primer sets should yield a single denaturation event. Regarding data normalization, several methods exist, namely the background subtraction (Mutskov and Felsenfeld, 2004), percent of input (% IP, Nagali *et al.,* 2003), fold enrichment (Tariq *et al.,* 2003), normalization relative to a control sequence (Mathieu *et al.,* 2005) and normalization relative to nucleosome density (Kristjuhan and Svejstrup, 2004). In this work, we followed the % IP method, in which the qPCR signals derived from the ChIP samples are divided by the qPCR signals derived from the input sample. Additionally, the background signal evidenced by the NoAb sample, is subtracted to the ChIP samples, according the formula % IP = (AB-NoAB)/input.

4.4. Results

4.4.1. The *OsRMC* gene activation in response to salt is epigenetic modulated

The expression level of the *OsRMC* gene was first analysed in leaf tissues of 14-days-old rice seedlings cv. Nipponbare in control conditions or after applying a salt treatment. The basal expression of *OsRMC* gene was very low under control conditions but the imposition of salt for 5 or 24h caused a significant increase in the *OsRMC* expression (Figure 1A) as previously reported by Serra *et al.* (2013). In this work, we investigated whether epigenetic mechanisms could have a role in regulating the expression of the *OsRMC* gene. First, *OsRMC* transcription was evaluated when seedlings were treated with the DNA hypomethylating agent 5-Azacytidine (5-AC) for 3 or 7 days. The 5-AC treatment caused an induction of *OsRMC* expression, particularly when the duration of the drug treatment was more extended (Figure 1B). The *OsRMC* expression was also analysed when epigenetic backgrounds are modified due to the presence of specific

mutations for epigenetic regulators. The *osdrm2* is a knockout rice mutant for a DNA methyltransferase required for *de novo* methylation (LOC_Os03g02010) and the *oshac704* mutant lacks one histone acetyltransferase (LOC_Os06g49130). The *OsRMC* expression was induced both in the WT and in the mutants after 24h of salt stress treatment but the mutants presented a higher *OsRMC* salt-induction than the WT and that was particularly evident in the *oshac704* mutant (Figure 1C). The statistical data analysis by two-way ANOVA revealed a significant interaction between gene and treatment ($F(2,12)=180.581$; $p<0.001$) as well as between treatments (control and salt) ($F(1,12)=1135.361$; $p<0.001$). These results indicate a link between epigenetic factors and *OsRMC* gene activation by salt stress.

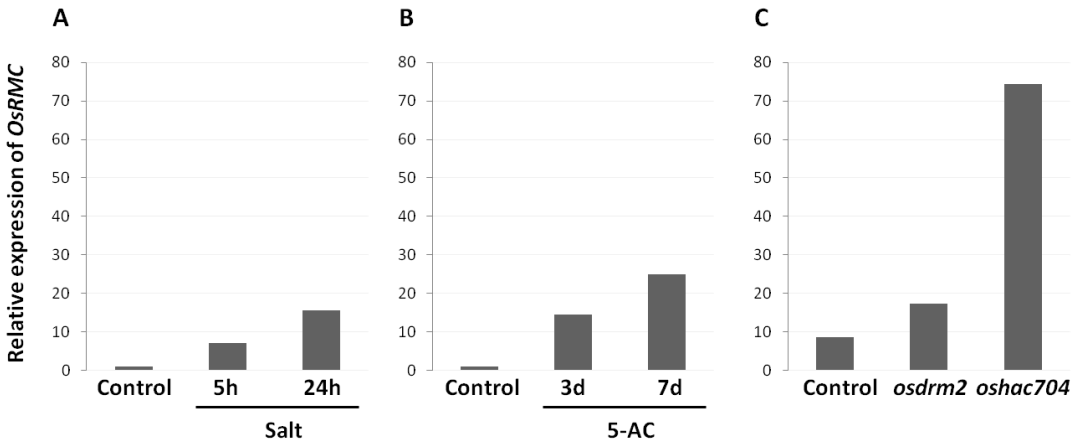

Figure 1: *OsRMC* expression studies. Semi-quantitative RT-PCR reactions were performed with cDNA prepared from total RNA extracted from leaves of 14-days-old seedlings of 'Nipponbare' subjected to high salinity (150 mM NaCl) treatment during 5 and 24h **(A)** or to the 5-azacytidine treatment (5-AC 80 µM) during 3 or 7 days **(B)**. The data were normalized to the internal control 25S rRNA. The graphics shown in (A) and (B) were made based on the gel bands analysis with Image J. **(C)** Quantitative real-time qPCR reactions were performed with cDNA prepared from total RNA extracted from leaves of 14-days-old seedlings of *osdrm2* and *oshac704* mutants and correspondent wt subjected to high salinity (150 mM NaCl) during 24h. The data were normalized to the internal control 25S rRNA. The mean expression values represent fold change in expression of salt over control conditions.

4.4.2. *In situ* imaging of histone modification marks revealed an enrichment in euchromatic marks in response to salt stress

3D imaging of histone modification marks at single-cell resolution can provide information regarding the dynamics of transcriptionally active and inactive regions in interphase nuclei. We previously showed that salt stress can induce decondensation of heterochromatic ribosomal chromatin (Santos *et al.,* 2011b). In this work we performed immunofluorescence with a specific antibody against H3K4me2 in meristematic cells from rice (cv. Nipponbare) root-tip sections from 3-days-old seedlings in control and salt stress conditions. The H3K4me2 is widely distributed through the entire nucleus, with some more densely marked areas at the perinucleolar domain (Figure 2A). The imposition of salt stress triggered an enrichment of the H3K4me2 mark, although the distribution pattern remained unaltered (Figure 2B). Moreover, the increasing of H3K4me2 levels in response to salt was also confirmed by western-blot analysis (Figure 2C).

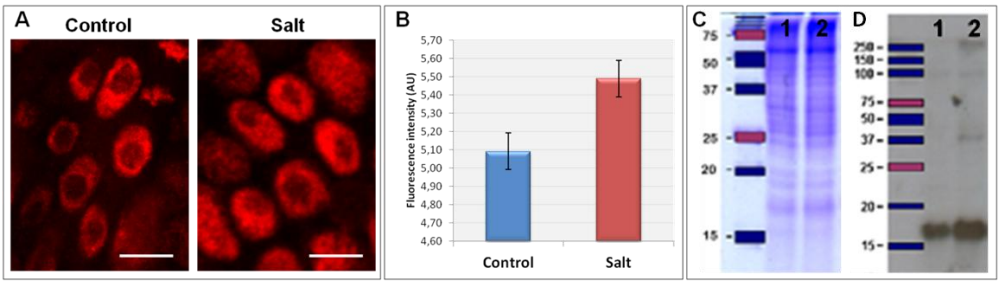

Figure 2: Genome wide detection of H3K4me2 is response to salt stress. (A) Immunofluorescence with a specific antibody against H3K4me2, a euchromatic marker, performed in single interphase nuclei of rice (cv. Nipponbare) root sections after seed germination in control conditions or in 150mM NaCl. Bar = 20 µM. **(B)** Fluorescence intensity was measured using Image J. The values obtained are shown as arbitrary units (AU). **(C)** Coomassie stained gel of rice root total proteins. **(D)** Immunodetection of H3K4me2 levels by western blot analysis. (1-control, 2-salt).

4.4.3. The enrichment of euchromatic histone marks correlates with the *OsRMC* activation in response to salt stress

The activation of genes has been frequently associated with specific histone modifications, namely with acetylation and methylation of specific histone residues. In order to investigate putative links between specific histone modification marks and the *OsRMC* induction by salt stress, we deciphered the histone landscape, specifically acetylation and methylation of histones H3 and H4 along the *OsRMC* gene promoter. Chromatin immunoprecipitation (ChIP) assays were carried out in non-stressed and in salt-stressed plants using antibodies that recognize distinct histone modification marks such as the H3K9ac, H4K5ac, and H4K20me3, all described as euchromatic marks in plants. Three specific regions of the promoter region of *OsRMC* gene were analysed as it is schematically represented in Figure 3A. The promoter region A covers the region -1159 to -1073 bp, upstream the ATG including also the binding site of the TFs OsEREBP1 and OsEREBP2 as identified by Serra *et al.* (2013). The promoter regions B and C are located in the regions -1079 to -967 bp and -773 to -693 bp upstream the ATG, respectively.

The distribution of specific histone modification marks was not homogenous along distinct *OsRMC* promoter regions (Figure 3B). The promoter region C, the closest to ATG, presented a higher enrichment in all the histone marks analysed as compared to the promoter regions more far away from ATG. The landscape of histone modifications was dynamics and salt stress responsive. Under control conditions, the histone marks present on the promoter region A were barely detected (Figure 3B). However, after 5h of salt treatment, there was an increase of the H4K5ac and H4K20me3 marks. The promoter region B, on the vicinity of the TFs binding site, was depleted of H4K5ac in control conditions but got an enrichement on this mark under salt stress (Figure 3B). On the contrary, the levels of H3K9ac and H3K20me3 marks decreased with salt stress. Concerning the promoter

of region C, all histone marks analysed were detected in high levels in control conditions but were drastically reduced upon salt stress (Figure 3B). All together, these results show a differential enrichment of euchromatic marks depending on the promoter region.

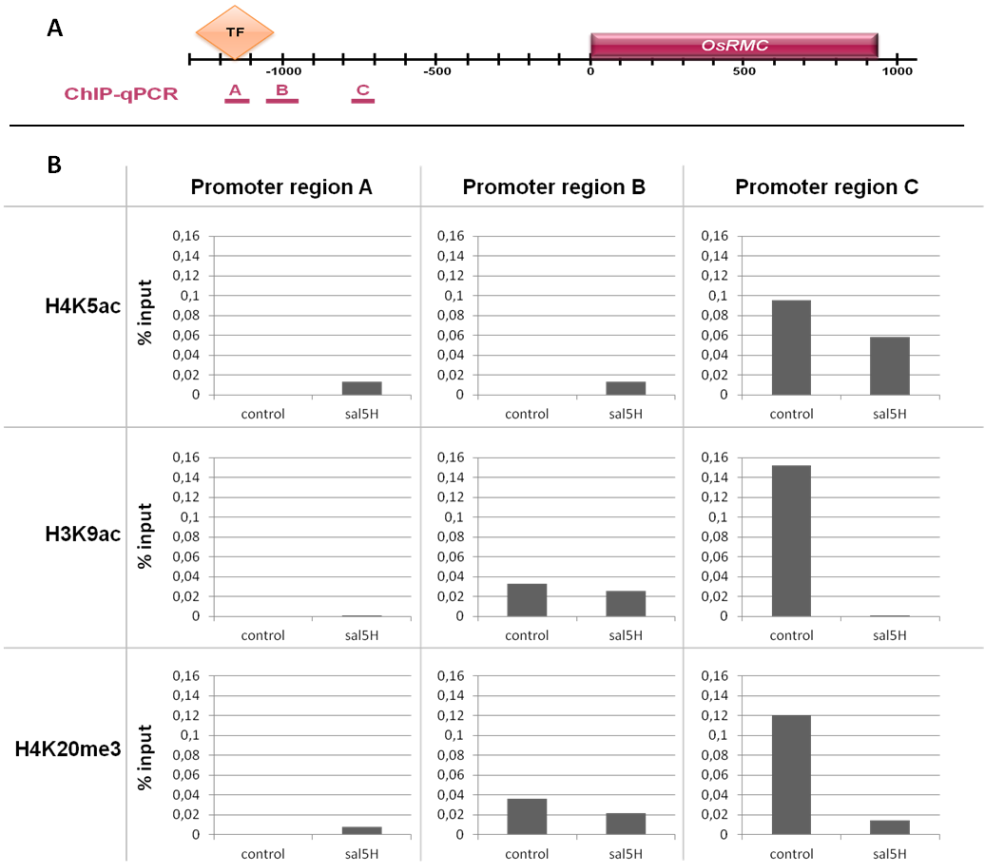

Figure 3: Dynamics of H3K4ac, H3K9ac and H4K20me3 marks at *OsRMC* gene promoter after salt stress. (A) Schematic representation of the *OsRMC* promoter regions analyzed: promoter region A [-1159; -1073], promoter region B [-1079; -967], promoter region C [-773; -693]. The diamond shape indicates the binding site of the transcription factors *OsEREBP1* and *OsEREBP2* as reported by Serra *et al.* (2013). **(B)** The levels of histone modification marks were determined by ChIP using specific antibodies for acetylation of histone H3 at the lysine 4 and 9 (H3K4 and H3K9) and for trimethylation of histone H4 at lysine 20 (H4K20). The samples were analyzed using real-time qPCR to quantify *OsRMC* gene promoter DNA enriched in the immunoprecipitates. Data was normalized following the % input method, in which the qPCR signals derived from the ChIP samples are divided by the qPCR signals derived from the input sample.

4.5. Discussion

High salinity is an abiotic stress condition able to trigger several responses in plants, including changes in the expression of several genes. The rice *ROOT MEANDER CURLING* (*OsRMC)* gene was shown to be highly activated in response to salinity stress (Serra *et al.,* 2013). The OsRMC protein is a receptor kinase that has been described as negative regulator of salt stress responses (Zhang *et al.,* 2009), meaning that it can repress the induction of salt responsive genes (Serra *et al.,* 2013). Thus, the *OsRMC* gene may have a key role in adjusting plant responses to salt stress. Epigenetic mechanisms participate in the regulation of gene expression and in this study we sought to investigate the role of specific histone modifications underlying the activation/repression of the salt stress-responsive *OsRMC* gene. Gene expression studies in rice mutants with altered epigenetic backgrounds revealed a salt stress induction of *OsRMC* particularly notorious in the *hac704* mutants, where one histone acetyltransferase is knocked-out. These results suggest a role of specific histone modifications in the regulation of *OsRMC* expression. To better understand this regulation we analysed the H3K9ac, H4K5ac and H4K20me3 marks along distinct *OsRMC* promoter regions. One promoter region analysed, identified as promoter region A, overlaps with the binding site of two transcription factors, OsEREBP1 and OsEREBP2, previously reported to be involved in the *OsRMC* regulation (Serra *et al.,* 2013). The histone marks analysed were not detected on promoter region A under control conditions, which may agree with the concept of nucleosome-free regions around TF binding sites (Wang *et al.,* 2011; Lenhard *et al.,* 2012; Ozonov and Nimwegen, 2013). In contrast, salt stress treatment was associated to an increase of the H4K5ac and H4K20me3 marks at promoter region A, suggesting a nucleosome repositioning triggered by salt stress. The occurrence of a nucleosome repositioning is reinforced by the observation that chromatin changes may also occur in the other promoter

regions analysed, namely a decrease of H3K9ac and H4K20me3 in the promoter region B. Interestingly, in the promoter region C, which is the one closest to the transcription starting site, all histone modification marks analysed were detected in higher amounts, and that may eventually suggest the inclusion of this promoter region in a distinct nucleosome. The OsEREBP1 and the OsEREBP2 TFs were described as transcriptional repressors (Serra *et al.,* 2013). Thus, the enrichment in euchromatic marks triggered by salt stress may well be needed for triggering chromatin unfolding and enabling TFs releasing, with the subsequent activation of *OsRMC* gene. Moreover, the TFs themselves may directly (or indirectly via co-regulators) be responsible for the recruitment of histone-modifying complexes. Several plant TFs are known to interact and recruit histone modifying complexes, including some involved in abiotic stress response (Pfluger and Wagner, 2007). For example, the cold-inducible C-REPEAT BINDING FACTOR (CBF1) recruits the GCN5 HAT complex via direct interaction with the HAT complex subunits ADA2a and ADA2b (Mao *et al.,* 2006). The APETALA2/EREBP-type transcription factor AtERF7, known to be involved in ABA responses, recruits HDA19 via its interaction with the HDA complex subunit SIN3 (Song *et al.,* 2005). In addition, the TFs OsEREBP1 and OsEREBP2 that bind to the *OsRMC* promoter, belong to the ERF subfamily of TFs (Serra *et al.,* 2013). These studies indicate that TFs likely play a crucial role in the establishment of *locus*-specific chromatin remodelling, essential for transcriptional regulation, directly affecting nucleosome structure with consequences in gene activation/repression.

In summary, this work deciphered chromatin changes along distinct regions of the *OsRMC* promoter, in response to salt stress.

In the model we propose that in control conditions the TFs remain bound to the *OsRMC* promoter blocking gene expression, while under salt stress euchromatic marks are enriched, TFs released and chromatin remodelers

recruited, thus reshaping nucleosome structure to a transcription-competent state (Figure 4).

All together, our results suggest a coordinated action of TFs and histone modifications in *OsRMC* transcriptional regulation. However, it remains to be proved whether TFs are released from the promoter binding site, and even whether they interact with chromatin remodelers. Higher resolution chromatin studies will hopefully contribute to a better understanding of this fine regulation.

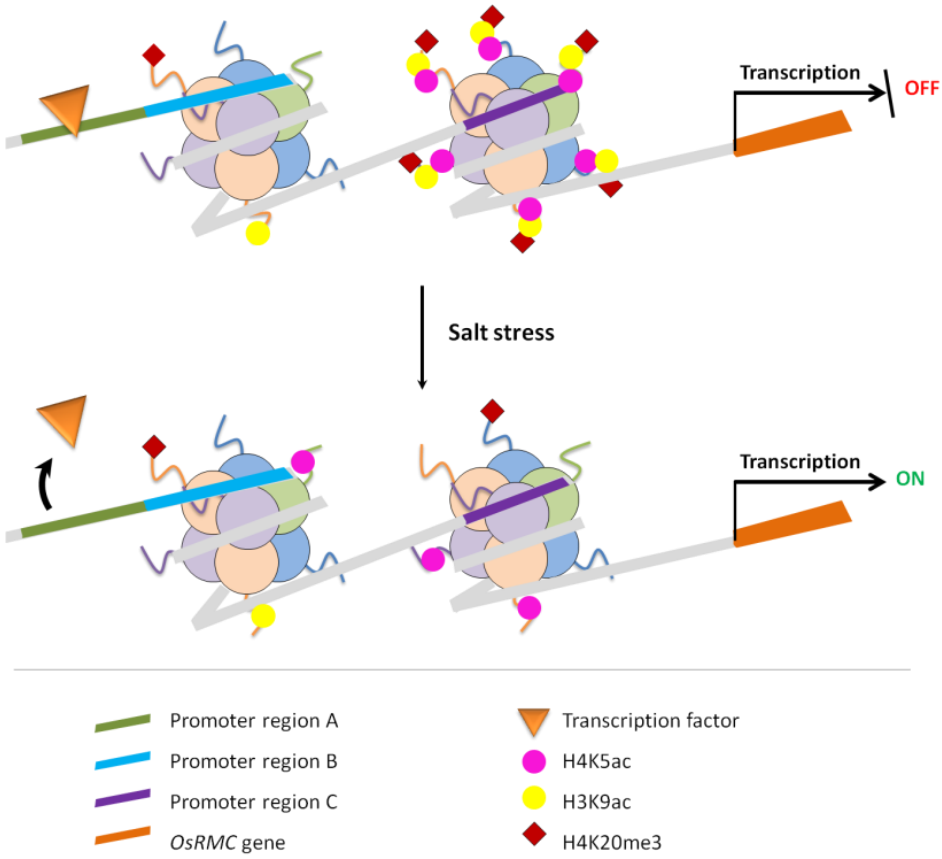

Figure 4: A proposed schematic model for explaining the role of epigenetic factors and chromatin dynamics on salt stress induction of *OsRMC* under salt stress. It is proposed that salt stress-induced *OsRMC* activation may involve the release of transcription factors from a nucleosome free promoter region together with an enrichment of specific euchromatic histone marks contributing to a chromatin conformation more amenable to gene transcription.

4.6. Acknowledgments

The authors thank Christoph Peterhänsel by technical help on establishing the ChIP protocol. The work was supported by the FCT (Portuguese Foundation for Science and Technology) through the project "Epigenetic regulation of the rice genome under environmental stresses" '[BIA-BCM/111645/2009]' and through the R&D unit, UID/Multi/04551/2013 (GREEN-IT). APS and LF were supported by FCT grants '[BPD/74197/2010]' and '[BD/61428/2009]', respectively.

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4.8. Supporting information

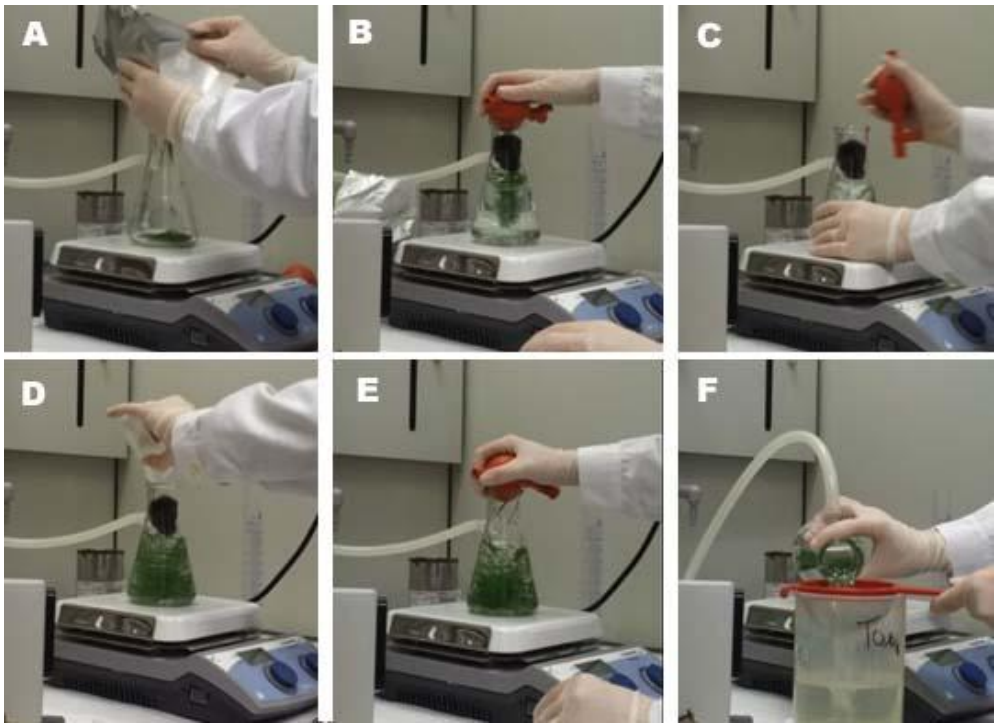

Figure S1: Visual description of the crosslink step in the ChIP process. (A) Leaf fragments with approximately 1 cm length are placed in a flask and immediately mixed with the formaldehyde solution (Buffer A). **(B)** The flask is covered for vacuum infiltration. **(C)** When pressure reaches 50 mbar, the vacuum is released and the cycle is repeated approximately 6-7 times during 2 min, followed by stirring an additional 1 min without vacuum **(D)** The crosslinking reaction is stopped by adding 20 ml of 2M Glycin. **(E)** Vacuum is agan applied for 5 min with pressure release every 30 s. **(F)** The leaves are then washed in water (using a sieve), carefully dried between paper towels, and finally frozen in liquid nitrogen until further use.

	CROSSLINKED			DE-CROSSLINKED		
Samples	CTs	[DNA]	Normaliz.	CTs	[DNA]	Normaliz.
0%	16,600	1,03E-05	1,000	22,417	1,85E-07	1,000
0.8%	23,993	6,21E-08	0,006	22,253	2,07E-07	1,120
1%	24,327	4,93E-08	0,005	24,237	5,25E-08	0,284

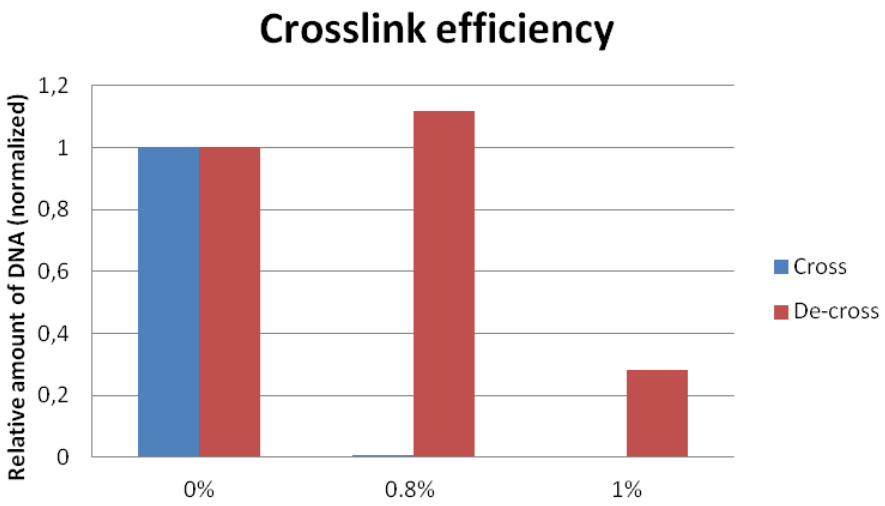

Figure S2: FAIRE assay to test chromatin crosslink efficiency. Two formaldehyde concentrations (0.8% and 1%) were compared against crosslinking with water (0%). Primers specific for the *OsUBC2* gene were used and their efficiency was calculated according the formula E=10(1/slope) (efficiency = 1.996933). This efficiency value was then used to estimate the initial concentration of DNA present in the samples, following the formula [DNA] = Efficiency-Ct. The concentrations were normalized against 0% formaldehyde values and plotted. Both formaldehyde concentrations assured a high degree of crosslinking, but DNA recovery after de-crosslink was higher when using 0.8% formaldehyde, reason why this formaldehyde concentration was selected for further assays.

Table S1: List of primers used for gene expression analysis of *OsRMC* gene.

Gene	Primer Forward	Primer Reverse
For qPCR		
OsRMC	TCTACAACGAGTGCTACGCC	CTGCTCATGAGGGTCTTCAG
25S rRNA	AAGGCCGAAGAGGAGAAAGGT	CGTCCCTTAGGATCGGCTTAC
For semi-quantitative RT-PCR		
OsRMC	CCTCCCAGGAGATCCTCTTC	GGGCGTAGCACTCGTTGTAG
25S rRNA	ATGATAACTCGACGGATCGC	CTTGGATGTGGTAGCCGTTT

Table S2: ChIP buffers

Buffer A	10 mM sodium butyrate 0.4 M sucrose 10 mM Tris (pH 8.0) 5 mM β-mercaptoethanol 0.1 mM PMSF 0.8% formaldehyde
Buffer B	10 mM sodium butyrate 0.4 M sucrose 10 mM Tris (pH 8.0) 5 mM β-mercaptoethanol 0.1 mM PMSF 1 M protease inhibitor
Buffer C	10 mM sodium butyrate 0.25 M sucrose 10 mM Tris (pH 8.0) 5 mM β-mercaptoethanol 0.1 mM PMSF 10 mM MgCl2 1 M Triton X-100 1 M protease inhibitor
Buffer D	10 mM sodium butyrate 1.64 M sucrose 10 mM Tris (pH 8.0) 5 mM β-mercaptoethanol 0.1 mM PMSF

	2 mM MgCl2 150 mM Triton X-100 1 M protease
Buffer E	25 mM Tris (pH 8.0) 5 mM EDTA 0.5 M SDS 0.1 mM PMSF 1 M protease inhibitor
Buffer F	50 mM Tris (pH 8.0) 1 mM EDTA 150 mM NaCl 100 mM Triton X-100
Buffer G	62.5 mM Tris (pH 6.8) 200 mM NaCl 2 M SDS 10 mM DTT
Low salt buffer	0.15 M NaCl 0.1 M SDS 1 M Triton X-100 2 mM EDTA 20 mM Tris (pH 8.0)
High salt buffer	0.5 M NaCl 0.1 M SDS 1 M Triton X-100 2 mM EDTA 20 mM Tris (pH 8.0)
LiCl buffer	0.25 M LiCl 0.1 M NP-40 24 mM sodium deoxycholate 1 mM EDTA 20 mM Tris (pH 8.0)
TE buffer	10 mM Tris (pH 8.0) 1 mM EDTA
Extraction buffer	0.1 M NaCl 0.5 M SDS 50 mM EDTA 100 mM Tris (pH 8.0)

Table S3: List of primers used for analysis of immunoprecipitated DNA by qPCR.

Promoter region (relative to ATG)	Primer Forward	Primer Reverse
A -1160 to -1074	TTGACGAGCAGGCATAGGTA	CTGGATTGTCTCGGTGGAAT
B -1080 to -968	ATCCAGTTCGTTGCCATCTC	CGGAATGAACGGTGATCCTA
C -774 to -694	GGCACAGATATCCCCTTTGA	CCGTGAGAGCCCATTTTTAC

Chapter 5

General Discussion

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General Discussion

The term epigenetics derives from Waddington's coining of the term "epigenesis" to capture his logical deduction that during organismal development a layer of mechanisms must surely exist that reside above (*epi*) the level of the genes, controlling their output in order to specify cell fate determination. Regarding the underlying biochemistry, there are two main epigenetic mechanisms, DNA methylation and regulation of chromatin structure via histone modifications. Epigenetics plays essential roles in cell differentiation, development and disease. Recent studies have indicated that stress responses include a substantial epigenetic control, with the regulation of stress responsive genes often being dependent on chromatin remodelling. Although the increasing number of evidences pointing for the involvement of epigenetic mechanisms on stress responses, the precise mechanisms underlying such regulation remains to be elucidated. In this thesis we described the epigenetic changes induced by salinity stress in rice, both at global and gene specific levels.

Rice is well suited to be used as a model organism to participate in the elucidation of epigenetic changes during salt stress responses since there are several genotypes with contrasting salt stress tolerance. Plants in general are good models for epigenetic studies since they can tolerate epigenetic mutations and still develop a phenotype, while in animals often these types of mutations are letal. Thus, although technically challenging, the identification of biologically relevant epigenetic changes is facilitated.

The first part of this thesis (Chapters 2 and 3) is dedicated to the study of methylome dynamics under salt stress. This investigation was done in a non-gene-specific manner simply asking how much does DNA methylation

patterns vary, among tissues and among rice genotypes, in control conditions as compared with imposed salinity stress. In chapter 2, we used an ELISA-based assay to determine relative global DNA methylation levels. This study revealed considerable differences between distinct tissues, with leaves showing significantly higher levels of DNA methylation than roots, which was evident in all genotypes analysed. Such tissue-dependent DNA methylation pattern may result from differences in tissue complexity and differentiation, namely the higher amount of undifferentiated meristematic tissue in roots. This hypothesis is supported by the fact that root methylation levels did not drastically change after salt stress imposition. Contrastingly, in leaves there was a clear association between demethylation and salt stress. Interestingly, this demethylation was smooth in the stress sensitive variety IR29 but more severe in the stress tolerant 'Pokkali'. Additionally, a phenotypic evaluation of some salt tolerance-related parameters, such as root length and biomass, revealed that the DNA methyltransferase osdrm2 mutant has better performance under stress. These findings emphasize a tissue- and genotype-specificity concerning global DNA methylation levels suggesting that higher methylome flexibility may be an important feature in salinity tolerance.

In chapter 3, the methylome of "Pokkali" was analysed by sequencing the methylated fraction of the genome (MeDIP-Seq), with the main goal of identifying differentially methylated regions between control and salt stress samples. Fifty three salt stress-specific differentially methylated regions (sDMRs) were identified. Further analysis of sDMRs genome location was performed to identify the genes in close proximity to DMRs. Interestingly, none of those neighbouring sDMRs correspond to known salt stress-responsive genes. However, the identification of new putative target genes may reveal novel players in plant salt stress responses. Transcription expression analysis confirmed the responsiveness of some of these genes

to salt stress imposition and further transcriptomic studies are aimed to cover the totality of the genes identified in these regions.

The last part of the thesis (Chapter 4) focused on the epigenetic regulation of *OsRMC*, a gene highly induced by salt stress and very conserved among rice varieties. The increased activation of *OsRMC* in the mutant line defective in one histone acetyltransferase led us to examine the landscape pattern and the dynamics of some histone modifications marks along specific *OsRMC* promoter regions. The ChIP analysis revealed a differential enrichment of euchromatic marks depending on the promoter region. The chromatin domain in which the transcription factors bind was particularly enriched in euchromatic marks after salt stress, possibly indicating a nucleosome-free region around the transcription factor binding site in control conditions, and a nucleosome repositioning after salt stress.

In conclusion, this work provides an important insight into the epigenetic regulation of the rice genome in response to salt stress. As summarized in Figure 1, the main thesis outputs include (1) the identification of a tissue- and genotype-specificity in the level of DNA methylation; (2) the establishment of an association between methylome flexibility and salt tolerance (Chapter 2). A deeper analysis of DNA methylation in the salt tolerant 'Pokkali' by MeDIP-seq enabled the identification of 53 salt stress-specific Differentially Methylated Regions between control and salt stress samples, likely playing a role as hotspots for gene regulation (Chapter 3). Moreover, the landscape profiling of histone modifications marks at specific *OsRMC* promoter regions allowed detecting a differential enrichment of euchromatic marks along the gene promoter, pointing for a nucleosome repositioning underlying gene activation (Chapter 4). All together, these results represent a step forward in the understanding of the role of epigenetic factors in plant response/tolerance mechanisms to salinity.

As future prospects, it should be emphasised the importance of methodologies allowing a cell-based resolution analysis. The isolation of

single cells can be currently achieved by droplet encapsulation, manual manipulation, fluorescence-activated cell sorting (FACS) or microfluidic processing (Clark *et al.,* 2016). Techniques involving super-resolution microscopy would be even more informative, since they would allow the in vivo analysis (Han *et al.,* 2013). Additionally, strategies allowing an integrative view of multiple epigenetic mechanisms may enhance our knowledge of genome regulation namely, on how epigenetic states can influence the phenotype.

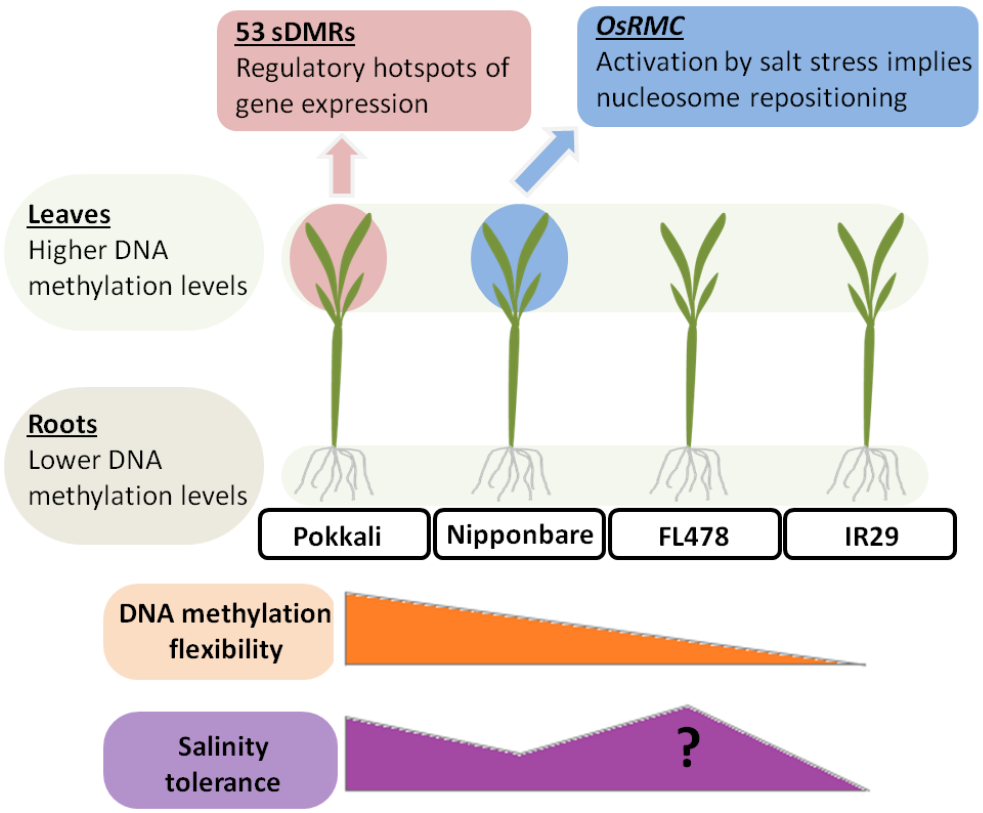

Figure 1: Salt stress-induced epigenetic changes in rice. The quantification of DNA methylation in leaves and roots of several rice varieties with distinct susceptibility to salinity revealed a global demethylation upon salt stress particularly evident in leaves. The salt tolerant variety Pokkali possesses a high methylome flexibility which may have a role in salinity tolerance. Tissue-specificity regarding global DNA methylation levels was clear since leaves have substantial higher amounts of DNA methylation than roots. In Pokkali, 53 salt stress-specific Differentially Methylated Regions (sDMRs) were identified between control and salt stress samples. Further research on sDMRs regulatory effects, namely on genes located nearby, may lead to discover novel players in plant salt stress responses. Finally, the histone modifications profile of the promoter region of the *OsRMC* gene allowed the detection of a differential enrichment of euchromatic marks, especially at the transcription factors binding site, and a nucleosome repositioning underlying *OsRMC* activation by salt stress.

5.1. References

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