Chapter 12

Deciphering Histone Modifications in Rice by Chromatin Immunoprecipitation (ChIP): Applications to Study the Impact of Stress Imposition

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Abstract

The spatial organization of chromatin, the methylome, and histone modifications represents epigenetic layers that greatly intersect each other, influencing genome regulation and allowing high flexibility in stress response. Although changes in specific histone modification marks could be extensively associated with transcriptional regulation of stress-responsive genes, a link between specific epigenetic signatures and plant stress tolerance has not yet been established. This chapter includes some examples of the associations found between fluctuations in these marks and regulation of plant stress-responsive genes. Chromatin immunoprecipitation (ChIP) has been widely used to uncover the landscape of histone modifications. However, ChIP involves multiple steps and requires optimizations targeting the tissue and the plant species. Here, we detail the ChIP procedure currently used in our laboratory, for leaf tissues of young rice seedlings, to decipher the dynamic feature of specific chemical modifications of histones that may influence the expression of stress-responsive genes. We show the success achieved after introducing specific optimizations and highlight the key critical steps and trouble shootings that may occur. A thorough understanding of stress-induced fluctuations of specific histone modifications may unveil new strategies to improve plant adaptation and performance in suboptimal conditions.

Keywords: abiotic stress, chromatin, epigenetics, histone modifications, rice

1. Introduction: histone modifications and gene expression regulation under stress

Rice is a very important crop whose production is being affected due to the climate changes we have been witnessing (higher and more variable temperatures, increased soil salinity, and extreme drought or flooding) have been negatively impacting rice production and sustainability [1]. Environmental stress experiences have been implicated in extensive changes of chromatin structure (e.g., decondensation of heterochromatic domains), and also in the plasticity of epigenetic marks [2, 3]. The flexibility of chromatin structure, chromatin loops, and epigenetic marks (DNA methylation and histone modifications) all play a role in gene expression regulation under stress [4]. This chapter focuses on the ChIP (chromatin immunoprecipitation) strategy to decipher the pattern of histone modifications particularly at specific promoter regions of selected stress-responsive genes. We describe in detail the ChIP protocol we are currently applying in our laboratory for leaf tissues of young rice seedlings.

At the basic level of chromosome structure, the DNA is bound to histone proteins forming the nucleosomes which represent the basic element of chromatin, being made of DNA around histones [5]. The histone tails can be modified by chemical groups, such as acetylation, methylation, phosphorylation, or ubiquitinylation, which can affect chromatin accessibility to the transcriptional machinery [6–8]. There are over 60 different residues on histones where chemical modifications have been detected and these posttranslational modifications (PTMs) of histones are the basis of a "histone code." The "histone code" was originally proposed by Strahl and Allis [9] and postulates that specific combinations of histone variants and PTMs can influence chromatin states [9]. For example, histone hyperacetylation has been generally correlated with transcription activation, while hypoacetylation has been associated with transcriptional silencing [9]. This biological effect is commonly explained by the fact that acetylation can lead to chromatin unwinding thus, decreasing their affinity for DNA and subsequently influencing the way transcription factors access DNA [10]. On the other hand, the presence of methyl groups at lysine residues can be reflected in different meanings including gene activation or repression, based on whether the lysine residue is mono-, di-, or trimethylated and also on which lysine residue is methylated [11]. Gene expression regulation can be greatly influenced by histone modifications landscape along the gene promoter region [12–14]. The analysis of histone modifications landscape has been mainly performed by chromatin immunoprecipitation which includes the cross-linking of histones and DNA, chromatin isolation and sonication, chromatin immunoprecipitation with antibodies specific for a given histone modification, de–cross-linking of histone-DNA complexes, DNA recovery, and gene-specific real-time quantitative polymerase chain reaction (PCR). Quantification of the relative proportion of the different loci to which the PTM is associated can be achieved by quantitative PCR (ChIP-qPCR) or microarray-based techniques (ChIP-chip), depending on the amount of loci one wants to analyze [15]. Large-scale enrichment analysis can also be performed through ChIP followed by DNA sequencing (ChIP-seq). This technique allows obtaining information regarding *in vivo* analysis of the protein-binding position in the

genome and thus can also be used if one wants to determine the specific targets of a given transcription factor.

In plants, environmental stress responses have been associated with the plasticity of histone modification marks, which in turn have been related to alterations in the expression of genes underlying responses to distinct stress types (**Table 1**). Nevertheless, the mechanistic links behind such connections are still largely unknown. This chapter is descriptive regarding the chromatin immunoprecipitation protocol that we are currently using to decipher the plasticity of histone modifications with particular focus on selected stress-responsive genes. The ChIP protocol we present refers to leaf tissues of young rice seedlings and is based on what was previously described for maize leaves [15] and thus, in its essence, is not new. However, here we describe important optimizations that take into account intrinsic specificities of rice leaf tissues. The whole procedure, from harvesting the rice leaves to the recovery of immunoprecipitated DNA, can be carried out within 3 days. The critical

Plant	Stress	Histone modifications	Biological effects	Reference
Rice	Submergence	↑H3 acetylation	↑ ADH1 and PDC1 stress responsive genes	[16]
Rice	Drought	↑H3K4me$_3$	↑ Dehydrin genes	[17]
Rice	Salt	↑H4K20me3 ↑H3K9ac, ↑H4K5ac	↑ OsRMC (salt-responsive gene)	This work (**Figure 4**)
Arabidopsis	Drought	↑H3K9ac ↑H3K4me3	↑ RD29A, RD29B, RD20,and RAP2.4 (drought-responsive genes)	[18]
Arabidopsis	Salt	↑H3K9K14ac ↑H3K4me3 ↓H3K9me2	↓ ABI1, ABI2, RD29A, RD29B, DREB2 (abiotic stress-responsive genes like)	[19]
Arabidopsis	Salt	↓ H3K27me3	HKT1 (Salt stress-responsive gene)	[20]
Arabidopsis	Dehydration	↑H3K4me1 ↑H3K4me2 ↑H3K4me3	↑Dehydration-responsive genes	[21]
Arabidopsis	Cold	↑H3 acetylation	↑CBF1	[22]
Arabidopsis	Cold	↑H3K27me3	↑COR15A and GOLS3 (cold-responsive genes)	[23]
Soybean	Salt	↑H3K4me3 ↑H3K9ac	Activation of members of AP2/EREB, bZIP, NAC and MYB transcription factors	[24]
Maize	Cold	↓H3 acetylation ↓H4 acetylation	↑Histone deacetylases	[25]

Table 1. Connections between plasticity of histone modifications marks and biological effects due to stress imposition.

steps and trouble shootings are clearly indicated along the procedure, including important optimizations made to improve cross-linking and increase sonication efficiency for young rice leaves. Regarding the antibody selection, ,to assess quality and specificity, we routinely perform immunodetection using distinct antibodies for histone modifications in rice root tissue sections. This *in situ* approach provides information regarding the spatial organization pattern of specific histone modifications in individual cells during interphase when transcription is intensely occurring. Very briefly, the procedure includes the vibratome sectioning of root tips of 3-day-old rice seedlings, three-dimensional (3D) *in situ* immunofluorescence on preserved tissue sections followed by confocal microscopy analysis; for details on this protocol, see [3].

2. ChIP protocol for rice young leaves

2.1. Materials

Reagents	Supplies	Equipment
p-Formaldehyde 37%	Kitasato	Stirrer
Sodium butyrate	Rubber tubes	Vacuum pump
Sucrose	Sieve	Barometer
Tris	Small paintbrush	Chronometer
β-Mercaptoethanol	Liquid nitrogen	Mortar and pestle
PMSF	50 ml, 15 ml falcon tubes	Rotating mixer (2 ml, 50 ml tubes)
Glycine	Small funnel	Cold room
Protease inhibitor	Miracloth	Centrifuge
Magnesium chloride	DNA purification kit (Roche)	Sonicator (Bioruptor, Diagenode)
Triton X-100	Paper towels	Incubator (65°C)
EDTA	1.5 ml tubes	Vortex mixer
SDS		
Sodium chloride		
DTT		
Protein-A agarose		
Lithium chloride		
Sodium deoxycholate		
NP-40		

2.2. Buffers and solutions

Buffers	Solutions
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
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**
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 $MgCl_2$, 1 M Triton X-100, 1 M protease inhibitor*
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 $MgCl_2$, 150 mM Triton X-100, 1 M protease inhibitor*
E	25 mM Tris (pH 8.0), 5 mM EDTA, 0.5 M SDS, 0.1 mM PMSF, 1 M protease inhibitor*
F	50 mM Tris (pH 8.0), 1 mM EDTA, 150 mM NaCl, 100 mM Triton X-100
G	62.5 mM Tris (pH 6.8), 200 mM NaCl, 2 M SDS, 10 mM DTT
Low salt	0.15 M NaCl, 0.1 M SDS, 1 M Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.0)
High salt	0.5 M NaCl, 0.1 M SDS, 1 M Triton X-100, 2 mM EDTA, 20 mM Tris (pH 8.0)
LiCl	0.25 M LiCl, 0.1 M NP-40, 24 mM sodium deoxycholate, 1 mM EDTA, 20 mM Tris (pH 8.0)
TE	10 mM Tris (pH 8.0), 1 mM EDTA

*The sucrose solution should be filtered and kept at 4°C.
**We use the protease inhibitor "complete," from Roche.

2.3. Procedure

A. Collection of plant material and tissue fixation

1. Harvest 3 g of young rice leaves (around 70 plants), cut them into pieces of approximately 1–2-cm length and put the leaves immediately, as loose as possible, in a vacuum flask with a stirrer.

   Notes:

   a. The ChIP assays were performed in 14-day-old leaves of rice (*Oryza sativa* L. ssp japonica AA, 2*n* = 24) cv. Nipponbare. Two days before applying stress, for example, salt or cold, the plants, grown in a hydroponic system inside glass tubes, were transferred to larger flasks for better manipulation and faster harvesting of leaves.

   b. Place a sponge on top of the flask to avoid losing leaves by the suction applied during vacuum.

2. Submerge leaves in 200 ml Buffer A (0.8 % p-formaldehyde). Vacuum infiltrate at room temperature for 2 min (meaning vacuum up to 50 mbar, a short release of vacuum and then repeating the cycle six to seven times), allowing penetration of fixative into leaf

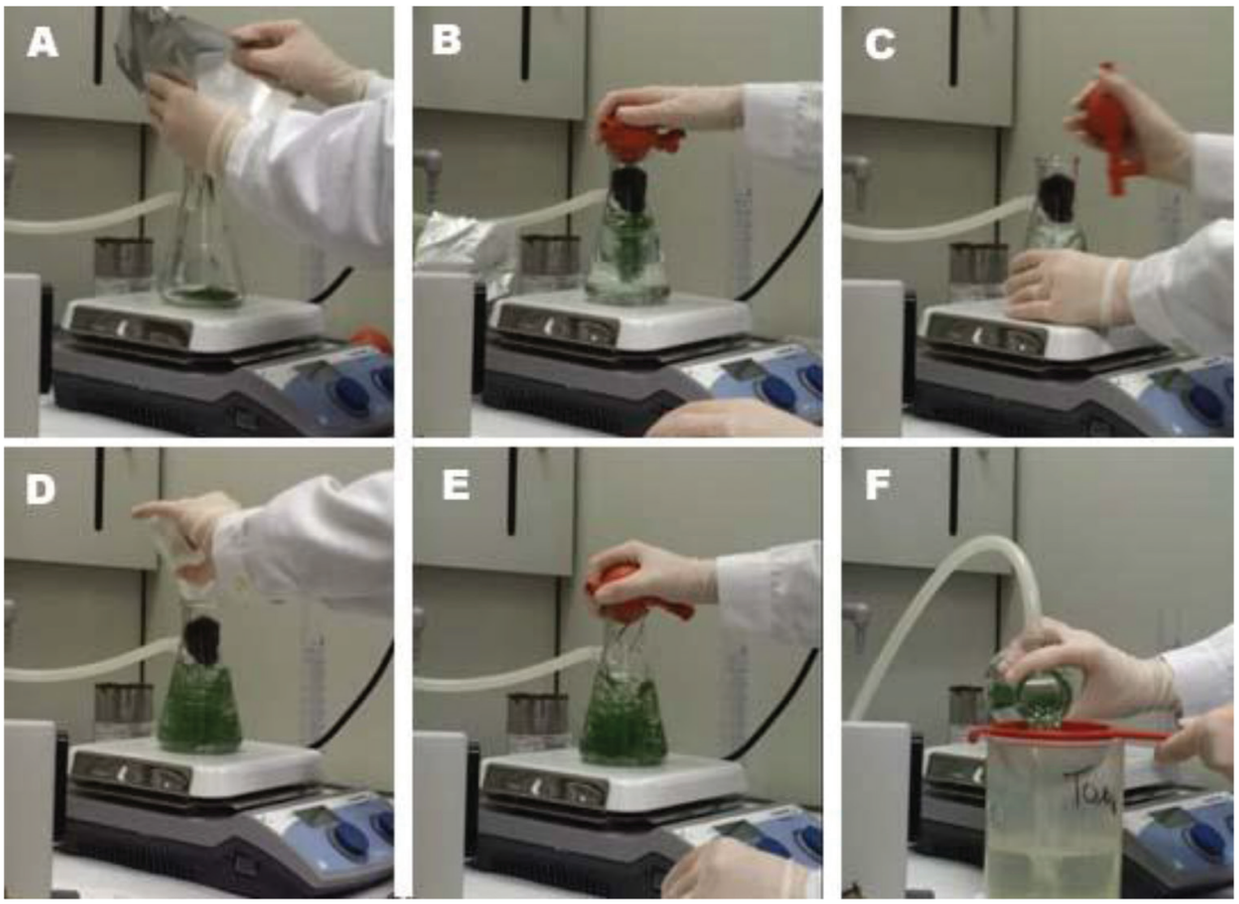

Figure 1. Schematic description of the cross-linking step in ChIP. (A) Leaf fragments with approximately 1-cm length are placed in a flask and immediately mixed with the formaldehyde solution (Buffer A). (B) The flask is covered for vacuum infiltration. (C) When pressure reaches 50 mbar, the vacuum is released and the cycle is repeated approximately 6–7 times for 2 min, followed by stirring an additional 1 min without vacuum. (D) The cross-linking reaction is stopped by adding 20 ml of 2M glycine. (E) Vacuum is again 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.

tissues. Stir for an additional 1 min without vacuum. Key steps of this procedure are depicted in **Figure 1**.

Notes:

a. A FAIRE (formaldehyde-assisted isolation of regulatory elements) test for assessing chromatin cross-link efficiency is routinely performed in our laboratory (**Figure 2**).

b. The color of the p-formaldehyde solution should be evaluated. A yellowish color indicates some oxidation and this will negatively affect the efficiency of the cross/de–cross-linking process.

c. When reducing the pressure to 50 mbar, one should see foam at the surface of the suspension; if not the vacuum gear insulation should be checked to make sure there is no leak. Also, ensure that leaf samples are fully submerged in solution A.

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	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

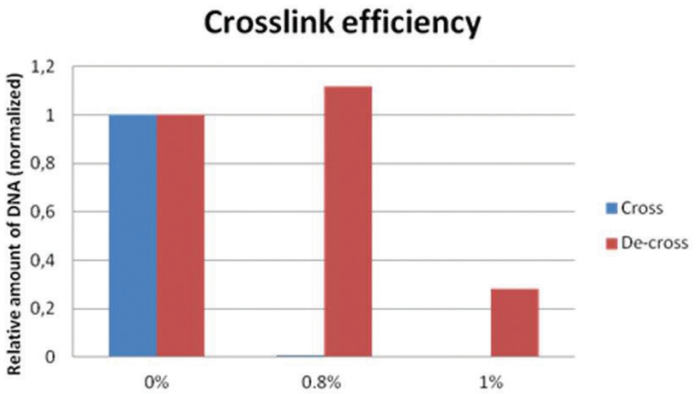

Figure 2. FAIRE assay to optimize chromatin cross-link efficiency. Two formaldehyde concentrations (0.8% and 1%) were tested against cross-linking with water (0%). Primers specific for the *OsUBC2* gene were used and their efficiency was calculated according to the formula $E = 10(1/\text{slope})$ (efficiency = 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 cross-linking, but DNA recovery after de–cross-link was higher under the use of 0.8% formaldehyde; thus, this formaldehyde concentration was elected as ideal for our material and assay conditions.

Caution: Formaldehyde is toxic and potentially carcinogenic, thus, particular precautions should be considered (e.g., working in the fume hood).

3. Add 20 ml glycine (2 M) to the flask, and mix vigorously to stop cross-linking. Vacuum infiltrate again during 5 min, releasing vacuum every 30 s.

4. Pour off the fixative solution and wash the leaves in a sieve with plenty of water. Dry the leaves carefully with paper towels and insert them into 50 ml tubes. Freeze in liquid nitrogen.

Pause point: Leaf tissue samples can be put in storage for several months at −80°C.

B. Isolation of nuclei and chromatin fragmentation

5. Grind the leaves, in liquid nitrogen (N_2), to a fine powder. Insert into 50 ml tubes and resuspend the ground material in 40 ml Buffer B.

   Note: Do not add the entire buffer at once. Tap the tube on the bench to get the N_2 out of the mix.

Caution: β-mercaptoethanol containing solution; work in the fume hood.

6. Incubate for 15 min at 4°C. Carefully, shake to release nuclei from cells (use a rotating mixer in a 4°C chamber).

7. Place a small funnel on top of a new 50 ml tube and filter the previous solution through it, using four layers of Miracloth.

   Note: Keep the tubes on ice.

8. Centrifuge the filtered solution for 20 min at 2880 × *g* at 4°C.

   Note: In the meantime, prepare Buffer C.

9. Gently, remove supernatant and resuspend the pellet in 1 ml Buffer C.

   Note: First, add 50 μl of Buffer C then, resuspend using a small paintbrush and then add the other 950 μl.

10. Transfer the solution to 1.5 ml Eppendorf tube and proceed to centrifugation at 12,000 × *g* for 10 min at 4°C.

    Note: In the meantime, prepare Buffer D.

11. Gently, remove supernatant and resuspend the pellet in 300 μl Buffer D.

    Note: First, add 50 μl of Buffer D, resuspend with a small paintbrush, and then add the other 250 μl.

12. Pipet 1.5 ml of Buffer D to a new 2 ml tube. Overlay this 1.5 ml of Buffer D with the resuspended pellet in the 2 ml tube.

    Note: The process should end up with a layer of extract (green) on top of the 1.5 ml Buffer D (colorless).

13. Centrifuge for 1 h at 16,000 × *g* at 4°C.

14. Remove supernatant and resuspend the chromatin pellet in 300 μl Buffer E.

    Note: Take a 30-μl aliquot for later gel analysis of "unsheared chromatin."

15. Sonicate the chromatin solution for successive cycles, 13 times, each time 30 s ON followed by 30 s OFF, selecting "LOW" power sonication, using the Bioruptor® Plus Sonicaton System, Diagenode. Water bath should be previously cooled to 4°C or less.

    Note: The shearing step is determinant on ChIP efficiency. To determine shearing efficiency, release bead-bound complexes from the sheared and unsheared samples by adding 100 μl Buffer G, vortex for 5 min, centrifuge briefly and incubate overnight at 65 °C. Purify these samples with a kit, eluting in 80 μl to get the most DNA possible out of the column, and then concentrate to ±15 μl with a speed vac. Add loading buffer and run on a 1.5% agarose gel. A DNA smear with 200–500-bp size range should be ideally obtained (see **Figure 3**).

16. Centrifuge the sonicated chromatin solution for 5 min at 16,000 × *g* at 4°C to sediment cell debris.

17. Take out the supernatant to a new tube and save again a 30 μl aliquot for gel analysis of "sheared chromatin."

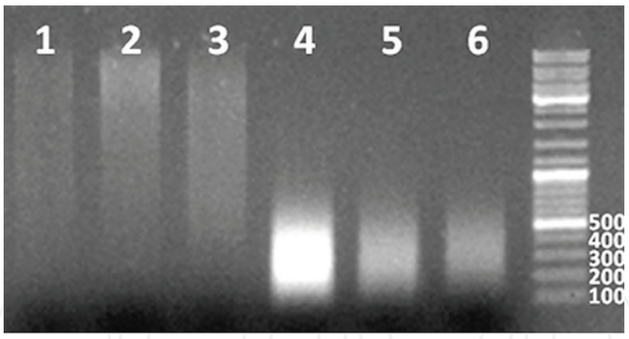

Figure 3. Chromatin fragmentation for ChIP. Unsheared (1, 2, 3) and sheared (4, 5, 6) chromatin samples. Sonication for 13 cycles, 30 s on and 30 s off, low-intensity sonication. The size of chromatin fragments was determined through agarose gel electrophoresis.

Pause point: The chromatin, once sonicated, can be kept at −80°C for a few months (not more than 3 months).

C. Preclearing

18. Use 200 μl chromatin solution and add 1.8 ml Buffer F and 30 μl protein-A agarose. Preclear the solution for 3 h at 4°C on a rotating mixer in the 4°C chamber.

19. Centrifuge at 4°C for 5 min at 500 × *g* and incubate for 5 min on ice. Collect the supernatant in a 2-ml tube and discard the beads.

D. Immunoprecipitation

20. Use the supernatant following this scheme:

Sample	Supernatant	Antibody	Protein-A Agarose
Antibody 1	400 μl	2–5 μl	30 μl
Antibody 1	400 μl	2–5 μl	30 μl
Antibody 1	400 μl	2–5 μl	30 μl
No Ab	400 μl	–	30 μl
Input	40 μl	–	–

Notes:

a. Freeze the input sample, as well as the supernatant that may be left.

b. Make sure to vortex the agarose beads prior to each use.

c. A successful ChIP assay depends on the quality of the antibody. In our laboratory, we routinely use ChIP-validated antibodies. Also, as a prior checking of the antibodies efficacy, we previously conducted in situ immunofluorescence in tissue sections and Western blotting analysis with commercial antibodies to histone modifications.

d. If the immunoprecipitation is inefficient or produces very low signals, the amount of antibody should be increased.

21. Incubate overnight at 4°C on a rotating mixer.

22. Pull down the agarose beads by centrifugation (5 min, 500 × *g*) and incubate on ice for 5 min. Collect the supernatant and add to the remainder chromatin solution frozen on step 20 and freeze it. This supernatant can be re-used to perform ChIP again. Proceed with the washing of the agarose beads.

E. Washes

23. Wash the beads using 900 μl buffer per wash followed by pelleting the beads (10 min on the rotating mixer, 4°C; spin 5 min at 500 × *g* and remove supernatant). Apply the washes in the following order: 1× low-salt wash buffer, 1× high-salt wash buffer, 1× LiCl wash buffer, 2× TE wash buffer.

    Note: The buffers should be prepared fresh. Remove TE totally after the final wash.

F. Reverse cross-linking

24. Release bead-bound complexes by adding 200 μl Buffer G, vortex for 5 min, centrifuge briefly, and incubate overnight at 65°C. Do this also with the "*input*" sample frozen on step 20.

25. Centrifuge shortly to sediment the agarose, collect supernatant (~100 μl), and purify it with a kit, eluting in 80 μl.

    Note: We use the high pure PCR product purification kit (Roche).

Pause point: ChIP samples, once purified, can be stored at −20°C for at least 1 month. Do not dilute prior to storage.

G. Quantitative PCR and data analysis

26. Using the ChIP-purified DNA, proceed to quantitative PCR using gene-specific primers.

    Notes:

    a. Real-time quantitative PCR was performed using the LightCycler 480 system (Roche). The PCR was carried out in a final volume of 20 μl containing 10 μl SYBR Green PCR Master Mix from Roche (2×), 5-μl ChIP DNA template, 1-μl primers (forward and reverse, 1 mM each, 3-μl sterile ddH_2O.

    b. qPCR 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.

    c. Several methods exist for data normalization, namely the background subtraction [26], percentage of input (% IP) [27], fold enrichment [28], normalization relative to a control sequence [29], and normalization relative to nucleosome density [30]. In our ChIP experiments, we used the % input method, in which the pPCR signals derived from the ChIP samples were divided by the qPCR signals from the input sample. Additionally,

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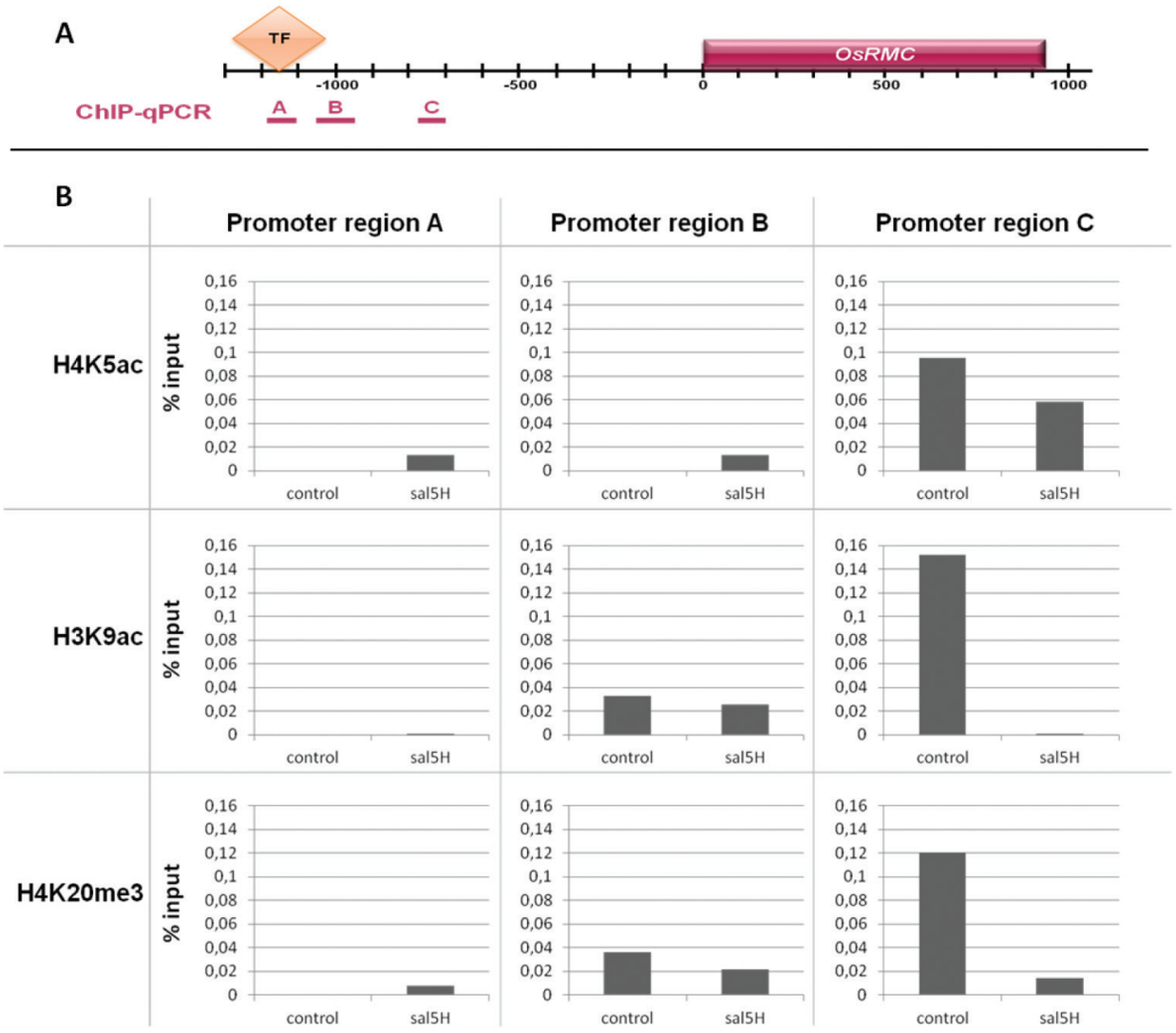

Figure 4. ChIP assay to determine the dynamics of H3K4ac, H3K9ac, and H4K20me3 marks at *OsRMC* gene promoter after salt stress. (A) Schematic representation of the *OsRMC* promoter regions analyzed: promoter region A [−1159; −1073], promoter region B [−1079; −967], and promoter region C [−773; −693]. Primer A forward TTGACGAGCAGGCATAGGTA, reverse CTGGATTGTCTCGGTGGAAT; primer B forward ATCCAGTTCGTTGCCATCTC, reverse CGGAATGAACGGTGATCCTA; primer C forward GGCACAGATATCCCC TTTGA, reverse CCGTGAGAGCCCATTTTTAC. The diamond shape indicates the binding site of the transcription factors *OsEREBP1* and *OsEREBP2* as reported by [31]. (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. The distribution of specific histone modification marks was not homogeneous along distinct *OsRMC*-promoter regions. The promoter region C, the closest to ATG, presented a higher enrichment in all the histone marks analyzed 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. However, after 5 h 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 enrichment on this mark under salt stress. On the contrary, the levels of H3K9ac and H3K20me3 marks decreased with salt stress. Concerning the promoter of region C, all histone marks analyzed were detected in high levels in control conditions but were drastically reduced upon salt stress. This example shows a differential enrichment of euchromatic marks dependent on the promoter region which may be interpreted from the viewpoint of gene expression regulation under stress.

the background signal evidenced by the NoAb sample subtracted to the ChIP samples, according to the formula % IP = (AB-NoAB)/input.

d. The analysis of immunoprecipitated DNA by qPCR enabled to evaluate the dynamics of specific histone modifications along specific regions of the OsRMC promoter under salt stress as exemplified in **Figure 4**.

3. Trouble shooting and future directions

The study of histone modifications has been mainly based on ChIP analyses, which is a very time-consuming process involving multiple stages. Some steps are particularly critical, such as cross-linking, sonication, and antibody immunoprecipitation, and must be previously optimized for each plant species and tissue. One critical point of the protocol concerns the formaldehyde cross-linking. With a low-efficiency cross-linking with formaldehyde, many DNA/protein interactions can be lost. On the other hand, if there is an excessive cross-linking, the DNA may not be recovered. Thus, various cross-linking times, as well as different formaldehyde concentrations, should be tested. Another possible problem is when a specific signal is observed but at very low levels (low input). That may happen because the chromatin structure itself may have been altered during the process and thus affecting the detection of specific regions. Also, the DNA-protein complexes may remain bound to the tubes during the procedure and in this case, the use of siliconized 1.5-ml tubes can help to solve this problem. A third critical point of the ChIP protocol refers to sonication efficiency since a deficient sonication can influence antigen accessibility that often results in a huge variability between experiments. In humans, some of these limitations have been overcome by combining ChIP procedure with microfluidic devices that in a semi-automated manner enables the identification of multiple marks, while requiring smaller volumes of samples and reagents and less human manipulation [32, 33]. This technology must still be extended to plants. At last, it must also be referred that ChIP studies have been mainly based on using heterogeneous cell populations which can lead to misleading results since epigenetic patterns are cell- and tissue-specific. Therefore, we anticipate a growing importance of methodologies allowing a cell-based resolution analysis of histone modifications. For such resolution, the isolation of single cells may be very important, and techniques such as droplet encapsulation, fluorescence-activated cell sorting (FACS), or microfluidic processing [34] are particularly relevant.

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